

PURIFICATION, KINETIC PROPERTIES, INHIBITION CHARACTERISTICS,
AND UNIQUE ACTIVE SITE FEATURES OF CARBONIC ANHYDRASE V

BY
ROBERT WILSON HECK

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1996

ACKNOWLEDGMENTS

This dissertation was made possible by the contributions of Dr. Sue Tanhauser, Rajyalakshmi Manda, and Ann Boriack-Sjodin. I feel grateful to many people who have supported and helped me throughout this process. Without their contributions, completion of this work would not have been possible.

I would like to extend many thanks to Dr. Philip J. Laipis, Dr. David N. Silverman, and Dr. C. K. Tu. Their guidance kept me on track and helped me focus on the important aspects. Having worked closely with them, I hope to take their words of wisdom with me and always strive to become a better researcher.

I would also like to thank Dr. Nancy Denslow and Dr. Thomas O'Brien. Their enthusiasm and love of science inspired my entry into research. They gave me a vision and taught me the methodology. I am forever grateful.

I was fortunate to have Dr. Daniel L. Purich and Dr. Richard Boyce to give me counsel and advice during times of difficulty. They helped me see the light at the end of the long tunnel. I would like to thank them for their insight

and for believing in me. Finally, special thanks go to Dr. Brian Cain and Dr. Maurice Swanson for their advice and patience.

TABLE OF CONTENTS

	<u>page</u>
ACKNOWLEDGMENTS.....	ii
LIST OF TABLES.....	vi
LIST OF FIGURES.....	vii
LIST OF ABBREVIATIONS.....	ix
ABSTRACT.....	x
 CHAPTERS	
1 INTRODUCTION.....	1
Carbonic Anhydrase Isozymes.....	1
Mitochondrial Carbonic Anhydrases.....	2
Comparison Of Carbonic Anhydrase V Sequence With Other Isozymes.....	4
Structure Of Carbonic Anhydrase V.....	8
Inhibition And Physiology Of Carbonic Anhydrase V.....	12
Enzymatic Mechanism.....	14
 2 PURIFICATION OF NATIVE CARBONIC ANHYDRASE V	 18
Introduction.....	18
Materials And Methods.....	20
Results.....	30
Discussion.....	35
 3 CLONING, MUTAGENESIS, SEQUENCING, EXPRESSION, AND PURIFICATION OF CARBONIC ANHYDRASE V.....	 39
Introduction.....	39
Materials And Methods.....	41
Results.....	52
Discussion.....	57

4	KINETIC AND INHIBITION PROPERTIES OF CARBONIC ANHYDRASE V.....	60
	Introduction.....	60
	Materials And Methods.....	62
	Results.....	65
	Discussion.....	67
5	PROTON TRANSFER AND THE EFFECTS OF ACTIVE SITE RESIDUES 64,65, AND 131 UPON THE CATALYTIC ACTIVITY OF CARBONIC ANHYDRASE V.....	73
	Introduction.....	73
	Materials And Methods.....	76
	Results.....	77
	Discussion.....	89
6	DISCUSSION AND CONCLUSIONS.....	94
	REFERENCES.....	101
	BIOGRAPHICAL SKETCH.....	106

LIST OF TABLES

<u>Table</u>	<u>Page</u>
2-1 Marker Enzyme Analysis of Mitochondria Preparation.....	31
2-2 R_1 and R_{H_2O} for Mouse Mitochondria and Mouse Blood in the Presence and Absence of Cupric Ions.....	31
2-3 Purification of Mitochondrial Carbonic Anhydrase.....	31
3-1 Oligonucleotides Used for the Cloning, Sequencing, and Mutagenesis of Mouse CA V.....	45
3-2 Expression Vectors of CA V and CA V Mutants.....	55
4-1 Maximal Values of the Steady-State Constants for the Hydration of CO_2 Catalyzed by Isozymes of Carbonic Anhydrase at $25^\circ C$	69
4-2 Inhibition of Isozymes of Carbonic Anhydrase Measured by ^{18}O Exchange at pH 7.2-7.5 and $25^\circ C$	69
5-1 Apparent pK_a and Maximal Values of k_{cat}/K_M Stopped Flow and ^{18}O Exchange Measurements of Catalysis by Mouse Carbonic Anhydrase V and Mutants of CA V.....	80
5-2 Apparent pK_a and Maximal Values of k_{cat} and R_{H_2O} for Mouse Carbonic Anhydrase V and Mutants of CA V.....	87

LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
1-1 Comparison of amino acid sequences for mouse, rat, and human carbonic anhydrase isozymes I, II, III, and V.....	5
1-2 Ribbon-type structure of the backbone of carbonic anhydrase V.....	9
1-3 Overlay of ribbon structures of CA V and CA II highlighting position 7 and position 64.....	11
2-1 Sequence comparison of rat and guinea pig CA V N-termini with the deduced amino acid sequences for mouse, human, and rat CA V.....	19
2-2 Stained blot of SDS-polyacrylamide gel electrophoretic separation of proteins from the purification of mitochondrial carbonic anhydrase.....	34
3-1 NH ₂ -terminal amino acid sequence of mouse CA V.....	42
3-2 Coomassie Blue-stained electrophoretic separation of purified recombinant CA V enzymes on a SDS-polyacrylamide gel.....	51
3-3 Nucleotide and deduced amino acid sequence for the coding region of CA V cDNA from BALB/c mice.....	53
4-1 k_{cat}/K_M catalytic constants for hydration of CO ₂ catalyzed by mouse carbonic anhydrase Vc and Vb.....	66
4-2 k_{cat} catalytic constants for hydration of CO ₂ catalyzed by mouse carbonic anhydrase Vc and Vb.....	68
5-1 ¹⁸ O exchange k_{cat}/K_M catalytic constants for hydration of CO ₂ catalyzed by mouse carbonic anhydrase Vc, mutants Y64A CA Vc, Y131A CA Vc, and Y64A/Y131A CA Vc.....	78

5-2	^{18}O exchange $k_{\text{cat}}/K_{\text{M}}$ catalytic constants for hydration of CO_2 catalyzed by mouse carbonic anhydrase Vc, mutants Y64H CA Vc, Y131H CA Vc, and Y64H/F65A CA Vc.....	79
5-3	Stopped-flow $k_{\text{cat}}/K_{\text{M}}$ catalytic constants for hydration of CO_2 catalyzed by mouse carbonic anhydrase Vc, mutants Y64A CA Vc, Y131A CA Vc, and Y64A/Y131A CA Vc.....	82
5-4	Stopped-flow $k_{\text{cat}}/K_{\text{M}}$ catalytic constants for hydration of CO_2 catalyzed by mouse carbonic anhydrase Vc, mutants Y64H CA Vc, Y131H CA Vc, and Y64H/F65A CA Vc.....	83
5-5	Stopped-flow k_{cat} catalytic constants for hydration of CO_2 catalyzed by mouse carbonic anhydrase Vc, mutants Y64A CA Vc, Y131A CA Vc, and Y64A/Y131A CA Vc.....	85
5-6	Stopped-flow k_{cat} catalytic constants for hydration of CO_2 catalyzed by mouse carbonic anhydrase Vc, mutants Y64H CA Vc, Y131H CA Vc, and Y64H/F65A CA Vc.....	88

LIST OF ABBREVIATIONS

CA, carbonic anhydrase
CHES, cyclohexylaminoethanesulfonic acid
cDNA, complementary DNA
Da, dalton
DNA, deoxyribonucleic acid
DNase, deoxyribonuclease
dNTPs, dATP + dCTP + dGTP + dTTP
dsDNA, double stranded DNA
E. coli, *Escherichia coli*
EDTA, ethylenediaminetetraacetic acid
g, gravitational force
HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IPTG, isopropylthio- β -D-galactopyranoside
 k_{cat} , turnover number
kDa, kilodalton
 K_M , Michaelis-Menten constant
l, liter
LB, Luria broth
MES, 2-(N-morpholino) ethanesulfonic acid
MSB, mannitol sucrose buffer
MW, molecular weight
MOPS, 3-(N-morpholino) propanesulfonic acid
NAD, nicotinamide adenine dinucleotide
NADH, reduced nicotinamide adenine dinucleotide
NH₂-terminus, amino terminus
Oligo, oligonucleotide
PEG, polyethylene glycol
 R_i , rate of interconversion of CO₂ and HCO₃⁻
 R_{H_2O} , rate of release of H₂¹⁸O from the enzyme
RNase, ribonuclease
SDS, sodium dodecyl sulfate
SDS-PAGE, polyacrylamide gel electrophoresis with sodium dodecyl sulfate
ssDNA, single stranded DNA
TAPS, 3-[[tris(hydroxymethyl)methyl]amino] propanesulfonic acid
TCA, trichloroacetic acid
TE, 10 mM Tris-HCL, 1 mM EDTA
Tris, tris(hydroxymethyl)aminomethane

Abstract of Dissertation Presented to the Graduate School of the University of
Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of
Philosophy

PURIFICATION, KINETIC PROPERTIES, INHIBITION CHARACTERISTICS,
AND UNIQUE ACTIVE SITE FEATURES OF CARBONIC ANHYDRASE V

By

Robert Wilson Heck

August 1996

Chairman: David N. Silverman

Major Department: Biochemistry And Molecular Biology

A cDNA encoding a new carbonic anhydrase isozyme reported by Amor-Gueret and Levi-Strauss (1990), CA V, was cloned and used to express active protein in *E. coli*. Purification and protein sequencing of the CA activity present in mouse liver mitochondria confirmed that the cDNA encoded a mitochondrial CA. Two potential mature forms were isolated, with N-terminal deletions of 29 or 51 amino acids. Two truncated CA V cDNAs, corresponding to the forms isolated from mitochondria, were created and used to express CA V in *E. coli*. These two forms of CA V had identical steady state constants for the hydration of CO₂, with $k_{cat}/K_M = 3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ and $k_{cat} = 3 \times 10^5 \text{ s}^{-1}$. These values are very similar to the rates determined for CA I and about 20% of the

values for CA II. CA V was inhibited by nanomolar concentrations of ethoxzolamide or acetazolamide, but was resistant to inhibition by micromolar concentrations of cupric ions.

The apparent pK_a of 9.2 for k_{cat} is much higher than seen in other isozymes. Substitutions of alanines or histidines for the tyrosines at positions 64 and 131 suggest that these tyrosines are not necessary for proton transfer. A mutant with a histidine at position 64 did not alter the high pK_a seen for k_{cat} although it did cause a slight increase in k_{cat} at low pH. The xray crystal structure of CA V indicated that a large phenylalanine at position 65 forces tyrosine 64 away from the active site. A double mutant with histidine 64 and alanine 65 had a maximal k_{cat} twice that seen for wild type CA V and a pH profile of k_{cat} that described two pK_a s of 6.3 and 9.0. This is the first example of a second proton shuttle being introduced into a carbonic anhydrase.

CHAPTER 1 INTRODUCTION

Carbonic Anhydrase Isozymes

Carbonic anhydrases (CA) are zinc containing metalloenzymes which catalyze the reversible hydration of CO_2 to form bicarbonate and a proton. The earliest reports of CA activity demonstrated that red blood cells contained a hemoglobin free extract that catalyzed the hydration of CO_2 (Meldrum and Roughton, 1933; Stadie and O'Brien, 1933). Purification of carbonic anhydrase from human blood resulted in the isolation of two distinct carbonic anhydrase isozymes (Nyman, 1961). These two isozymes were originally named carbonic anhydrase B and carbonic anhydrase C. Following the purification of a third isozyme of carbonic anhydrase from muscle tissue (Hölmes, 1977), the isozymes were designated by Roman numerals. CA B and CA C are now named CA I and CA II. The muscle isozyme is now designated as CA III.

To date, at least seven different isozymes of carbonic anhydrase are known to exist in amniotes, mostly from mammals (Tashian, 1989). These isozymes exhibit a remarkable diversity in tissue distribution, subcellular location, enzymatic activity, and inhibition properties. CA II is a cytosolic

enzyme found in nearly every tissue, while CA VI is secreted and found only in the salivary glands. There are even carbonic anhydrases found in plant chloroplasts which have completely unrelated amino acid sequences (Tashian *et al.*, 1991) and appear to be an example of convergent evolution.

Mitochondrial Carbonic Anhydrases

A carbonic anhydrase activity associated with mitochondria was noted in early reports (Datta and Shepherd, 1959; Maren and Ellison, 1967). The physiological function of mitochondrial carbonic anhydrase was initially suggested from the observation of decreased synthesis of urea and glucose in chameleons and alligators treated with CA inhibitors (Coulson and Herbert, 1984). Many subsequent studies (reviewed by Dodgson, 1991a) have suggested that a mitochondrial carbonic anhydrase activity is used in the metabolic pathways of gluconeogenesis and ureagenesis, both of which require bicarbonate as substrate for enzymes compartmentalized within the mitochondria. Although many of these studies postulated a unique mitochondrial form of carbonic anhydrase, the first evidence for a specific mitochondrial isozyme came from characterization of a protein purified from guinea pig liver mitochondria by Hewett-Emmett *et al.* (1986). They reported

an amino acid sequence of 24 residues with distinct similarities to other carbonic anhydrases (this isoform was subsequently termed CA V).

More recently, Amor-Gueret and Levi-Strauss (1990), while screening a mouse liver cDNA library for mRNA clones containing a mouse B2 repeat, isolated a cDNA which encoded a protein with strong sequence similarities to a carbonic anhydrase. Messenger RNA was detectable only in the liver when seven tissues were tested by Northern blotting and hybridization (Amor-Gueret and Levi-Strauss, 1990). Examination of this cDNA sequence and comparison with the partial sequence of Hewett-Emmett *et al.* (1986) suggested that the cDNA encoded the mouse homolog of the guinea pig mitochondrial carbonic anhydrase (CA V) with the addition of a potential mitochondrial leader sequence. Cloning and expression of the carbonic anhydrase V cDNA homologues from mouse, human, and rat liver mRNA has shown that CA V is targeted to and processed in the mitochondria of COS cells (Nagao *et al.*, 1994). Immunoblot analysis with antibodies raised against recombinant CA V in mouse and rat tissues shows that CA V is primarily found in liver, although immunoreactive bands are seen in all of the rat tissues tested (Nagao *et al.*, 1994). Immunological studies with polyclonal antibodies raised against a CA V extract prepared from mitochondria have also placed CA V in rat heart,

gastric parietal cell, and kidney intercalated cell mitochondria (Väänänen *et al.*, 1991; Karhukorpi *et al.*, 1992).

Comparison of Carbonic Anhydrase V Sequence with Other Isozymes

A comparison of the deduced amino acid sequences of carbonic anhydrase V with other isozymes of carbonic anhydrase (Fig. 1-1) shows that there is a high degree of sequence similarity. Mouse carbonic anhydrase V has 70%, 86%, 45%, 49%, and 43% sequence identity with the human CA V, rat CA V, mouse CA I, mouse CA II, and mouse CA III, respectively. The most highly conserved regions are found near residues which surround the active site, especially the histidines that coordinate the zinc ion (94, 96, 119) and threonine 199.

There are several regions of carbonic anhydrase V that differ significantly from the other isozymes. The most noticeable is the addition of 30-40 residues at the amino terminal end. This region has many of the structural properties of the mitochondrial targeting sequences collated by Hartl *et al.* (1989). It is rich in positively charged residues, lacks acidic residues (with the exception of a single aspartate in the fifth residue of mouse CA V), and has the potential to form an amphipathic helix.

					1	7
Musca5		.MLRRDPRKP	LAILRHVGLL	CATGPQRWRF	QHSAAEHSN	
Ratca5		.MLRA	KMLGRGPYKP	LAILRHMGPL	CATRPQHWRP	QHSYAEKHSN
Humca5		.MLGRN	TWKTSAFSFL	VEQMWAPLWS	RSMRPGRWCS	QRSCANQTSN
Musca3						.MAKEWGYAR
Ratca3						.MAKEWGYAS
Humca3						.MAKEWGYAS
Humca1						MASPDWGYDD
Musca1						MASADWGYGS
Musca2						.MSHHWGYSK
Ratca2						.MSHHWGYSK
Humca2						.MSHHWGYGK
	10	20	30	40	50	
Musca5	CARHPLWTGP	VSSAEGTRQS	PINIQWKDSV	YDPQLAPLRV	SYDAASCRL	
Ratca5	CARHPLWTGP	VSSPGGTQOS	PINIQTWDSV	YDPKLAPLRV	SYDAASCRL	
Humca5	NTLHPLWTVP	VSVPGGTRQS	PINIQRWDSV	YDPQLKPLRV	SYEAAACLYI	
Musca3	HNGPDHWHEL	YPIAKGDNQS	PIELHTKDIK	HDPSLQPWSV	SYDPGSAKTI	
Ratca3	HNGPEHWHEL	YPIAKGDNQS	PIELHTKDIR	HDPSLQPWSV	SYDPGSAKTI	
Humca3	HNGPDHWHEL	FPIAKGENQS	PVELHTKDIR	HDPSLQPWSV	SYDGGSAKTI	
Humca1	KNGPEQWSKL	YPIANGNNQS	PVDIKTSETK	HDTSLKPISV	SYNPATAKEI	
Musca1	ENGPDQWSKL	YPIANGNNQS	PIDIKTSEAN	HDSSLKPLSI	SYNPATAKEI	
Musca2	HNGPENWHKD	FPIANGDRQS	PVDIDTATAH	HDPALQPLLI	SYDKAASKSI	
Ratca2	SNGPENWHKE	FPIANGDRQS	PVDIDTGTAQ	HDPSLQPLLI	CYDKVASKSI	
Humca2	HNGPEHWHKD	FPIAKGERQS	PVDIDTHTAK	YDPSLKPLSV	SYDQATSLRI	
	60	70	80	90	100	
Musca5	WNTGYFFQVE	FDDSCEDSGI	SGGPLGNHYR	LKQFHFHWGA	TDEWGSEHAV	
Ratca5	WNTGYFFQVE	FDDSCESGI	SGGPLGNHYR	LKQFHFHWGA	TDEWGSEHMV	
Humca5	WNTGYLFQVE	FDDATEASGI	SGGPLENHYR	LKQFHFHWGA	VNEGGSEHTV	
Musca3	LNNGKTCRVV	FDDTYDRSML	RGGPLSRPYR	LRQFHLHWGS	SDDHGSEHTV	
Ratca3	LNNGKTCRVV	FDDTFDRSML	RGGPLSGPYR	LRQFHLHWGS	SDDHGSEHTV	
Humca3	LNNGKTCRVV	FDDTYDRSML	RGGPLPGPYR	LRQFHLHWGS	SDDHGSEHTV	
Humca1	INVGHFSFHV	FEDNDNRSVL	KGGPFSDSYR	LFQFHFHWGS	TNEHGSEHTV	
Musca1	VNVGHFSFHV	FDDSSNQSVL	KGGPLADSYR	LTQFHFHWGN	SNDHGSEHTV	
Musca2	VNNGHSFNVE	FDDSQDNAVL	KGGPLSDSYR	LIQFHFHWGS	SDGQGSEHTV	
Ratca2	VNNGHSFNVE	FDDSQDFAVL	KEGPLSGSYR	LIQFHFHWGS	SDGQGSEHTV	
Humca2	LNNGHAFNVE	FDDSQDKAVL	KGGPLDGTyr	LIQFHFHWGS	LDGQGSEHTV	

Figure 1-1. Comparison of amino acid sequences for mouse, rat, and human carbonic anhydrase isozymes I, II, III, and V. Numbering is based on human CA I (humca1).

	110	120	130	140	150
Musca5	DGHTYPAEHL	LVHWNSTKYE	NYKKASVGEN	GLAVIGVFLK	LGAAHQALQK
Ratca5	DGHAYPAELH	LVHWNMSKYE	NYKKATTGEN	GLAVIGVFLK	LGAAHHEALQR
Humca5	DGHAYPAELH	LVHWNMSVKYQ	NYKEAVVGEN	GLAVIGVFLK	LGAAHQTLQQR
Musca3	DGVKYAAELH	LVHWN.PRYN	TFGEALKQPD	GIAVVGILLK	IGREKGEFQI
Ratca3	DGVKYAAELH	LVHWN.PKYN	TSEEALKQPD	GIAVVGIFLK	IGREKGEFQI
Humca3	DGVKYAAELH	LVHWN.PKYN	TFKEALKQPD	GIAVVGIFLK	IGHENGFEQI
Humca1	DGVKYSAEHL	VAHWNSAKYS	SLAEAASKAD	GLAVIGVLMK	VGEANPKLQK
Musca1	DGTRYSGELH	LVHWNMSAKYS	SASEAISKAD	GLAILGVLMK	VGPANPSLQK
Musca2	NKKKYAAELH	LVHWN.TKYG	DFGKAVQQPD	GLAVLGYFLK	IGPASQGLQK
Ratca2	NKKKYAAELH	LVHWN.TKYG	DFGKAVQHPD	GLAVLGIFLK	IGPASQGLQK
Humca2	DKKKYAAELH	LVHWN.TKYG	DFGKAVQQPD	GLAVLGIFLK	VGSAPKFLQK
	160	170	180	190	200
Musca5	LVDVLPEVRH	KDTQVAMGPF	DPSCLPACR	DYWTYPGSLT	TPPLAESVTW
Ratca5	LVDILPEVRH	KDTQVTMGPF	DPSCLLPACR	DYWTYPGSLT	TPPLAESVTW
Humca5	LVDILPEIKH	KDARAAMRPF	DPSTLLPTCW	DYWTYAGSLT	TPPLTESVTW
Musca3	LLDALDKIKT	KGKEAPFTHF	DPSCLPACR	DYWTYHGSFT	TPPCEECIVW
Ratca3	LLDALDKIKT	KGKEAPFNHF	DPSCLPACR	DYWTYHGSFT	TPPCEECIVW
Humca3	FLDALDKIKT	KGKEAPFTKF	DPSCLPACR	DYWTYQGSFT	TPPCEECIVW
Humca1	VLDALQAIKT	KGKRAPPFTNF	DPSTLLPSSL	DFWTYPGSLT	HPPLYESVTW
Musca1	VLDALNSVKT	KGKRAPPFTNF	DPSSLLPSSL	DYWTYFGSLT	HPPLHESVTW
Musca2	VLEALHSIKT	KGKRAAFANF	DPCSLLPGNL	DYWTYPGSLT	TPPLLECVTW
Ratca2	ITEALHSIKT	KGKRAAFANF	DPCSLLPGNL	DYWTYPGSLT	TPPLLECVTW
Humca2	VVDVLDISIKT	KGKSADFTNF	DPRGLLPESL	DYWTYPGSLT	TPPLLECVTW
	210	220	230	240	250
Musca5	IVQKTPEVVS	PSQLSMFRTL	LFSGRGEEED	VMVNNYRPLQ	PLRDRKLRRS
Ratca5	IVHKMPIEVS	PSQLSTFRTL	LFSGRGEDEE	VMVNNFRPLQ	PLRGRNVRSS
Humca5	IIQKEPVEVA	PSQLSAFRTL	LFSALGEEEK	MMVNNYRPLQ	PLMNRKVWAS
Musca3	LLLKEPMTVS	SDQMAKLRLS	FSSAENEPV	PLVGNWRPPQ	PVKGRVVRAS
Ratca3	LLLKEPMTVS	SDQMANVRSL	FASAENEPV	PLVGNWRPPQ	PIKGRVVRAS
Humca3	LLLKEPMTVS	SDQMAKLRLS	LSSAENEPV	PLVSNWRPPQ	PINNRRVVRAS
Humca1	IICKESISVS	SEQLAQFRSL	LSNVEGDNAV	PMQHNNRPTQ	PLKGRTVRAS
Musca1	VICKDSISLS	PEQLAQLRGL	LSSAEGESAV	PVLSNHRPPQ	PLKGRTVRAS
Musca2	IVLREPITVS	SEQMSHFRTL	NFNEEGDAEE	AMVDNWRPAQ	PLKNRKIKAS
Ratca2	IVLKEPITVS	SEQMSHFRLK	NFNSEGEAEE	LMVDNWRPAQ	PLKNRKIKAS
Humca2	IVLKEPISVS	SEQVLKFRKL	NFNGEGEPEE	LMVDNWRPAQ	PLKNRQIKAS
	260				
Musca5	FRLDRTKMRS				
Ratca5	FQVPRVGTKS				
Humca5	FQATNEGTRS				
Musca3	FK.....				
Ratca3	FK.....				
Humca3	FK.....				
Humca1	F.....				
Musca1	F.....				
Musca2	FK.....				
Ratca2	FK.....				
Humca2	FK.....				

Figure 1-1 (continued)

Amino terminal sequencing of CA V purified from guinea pig and rat mitochondria suggests that not only are these extra amino terminal residues not present in the mature form of CA V, but that an additional 20 residues, corresponding to the amino termini of the other CA isozymes, are also removed (Hewett-Emmett *et al.* 1990, and Ohlinger *et al.*, 1993). This result is surprising since the tyrosine at position 7 of CA I, II, and III is thought to form a hydrogen bond with water in the active site of these enzymes (Ericksson *et al.*, 1986, Ericksson, 1988). However, carbonic anhydrase V lacks this conserved tyrosine and has a histidine (mouse and rat CA V) or a threonine (human CA V) at position 7. Tashian *et al.* (1991) have identified a partially conserved three-amino acid motif found in mitochondrial leader peptides that undergo a two step cleavage during import (Hendrick *et al.*, 1989) in the sequence for mouse carbonic anhydrase V. This motif predicts a cleavage after position 21 that would create a mature form of CA V that corresponds to the experimentally determined amino termini of guinea pig and rat CA V.

There is also some evidence that the region of CA V corresponding to residues 1-21 of CA I, II, and III is not removed. Ngao *et al.* (1994) have expressed human, mouse, and rat CA V in COS cells and found that these CA Vs, when purified from the mitochondria of the COS cells, have amino termini that begin at positions -3, -1, and 1, respectively. These sites are all preceded

by an arginine two residues into the leader sequence as is found in 74% of matrix protease cleavage sites (Hendrick *et al.*, 1989). A shortened form of human CA V with an amino terminus corresponding to that found for guinea pig and rat CA V was also extracted in the absence of protease inhibitors by Ngao *et al.* (1993), leading to the suggestion that the shorter forms are the result of proteolytic degradation during isolation.

The carboxy terminus of carbonic anhydrase V also has seven additional residues not found in isozymes I, II, and III. The purpose of these residues is not known. Other notable sequence differences are more easily described by comparing the three dimensional structure of the isozymes of carbonic anhydrases.

Structure Of Carbonic Anhydrase V

Boriack *et al.* (1995) have recently determined the structure of a truncated form of mouse carbonic anhydrase V lacking the putative mitochondrial leader sequence and residues corresponding to positions 1-21 (Fig. 1-2). This structure is very similar to the reported structures for human CA I and CA II (Eriksson *et al.*, 1986) and for bovine CA III (Eriksson, 1988) as would be expected from the high degree of sequence homology seen between all of these isozymes. The central portion of carbonic anhydrase V as well as

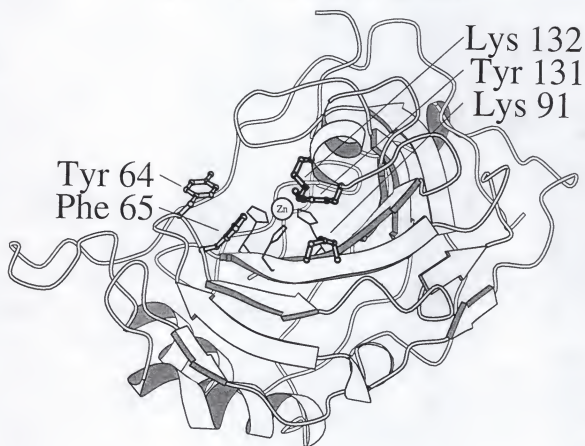


Figure 1-2. Ribbon-type structure of the backbone of carbonic anhydrase V.

CAs I-III is composed of a prominent β -saddle that enfolds the active site. The catalytic center of the active site is composed of a divalent zinc ion tetrahedrally liganded by three histidine residues at positions 94, 96, and 119 and a hydroxide ion. In terms of backbone atoms, all of these carbonic anhydrases have nearly identical structures near the active site.

An important feature of the active site of carbonic anhydrase II is the presence of a histidine at position 64. Tu and Silverman (1989) have shown that this residue functions as an intramolecular proton shuttle necessary for the high rate of catalysis seen in CA II. A histidine is also found at position 64 for CA I, IV, VI, and VII as well. CA III, which has significantly lower activity than the other isozymes, has a lysine at this position. Mutagenesis of lysine-64 to histidine-64 in HCA III results in a significant increase in enzymatic activity (Jewel *et al.*, 1991). Carbonic anhydrase V has a tyrosine at position 64. If this tyrosine is utilized as a proton transfer site, it should function differently than the histidine found in the other isozymes.

CA V is also unusual in that it has a bulky phenylalanine-65 (mouse and rat) or a leucine-65 (human) adjacent to tyrosine-64. All of the other isozymes have serine (CA I, IV, and VII), threonine (CA III and VI) or alanine (CA II) in this position. The xray crystal structure for mouse CA V (Boriack *et al.*, 1995) shows that this phenylalanine sterically hinders tyrosine-64 resulting in an

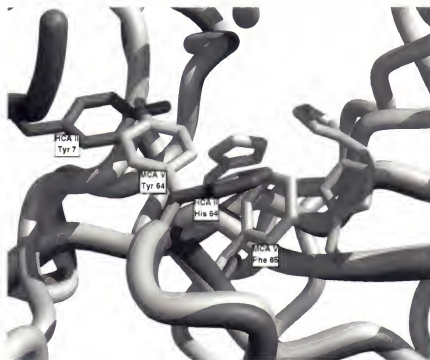


Figure 1-3. Overlay of ribbon structures of CA V and CA II highlighting position 7 and position 64

orientation away from the active site. The hydroxyl group of tyrosine-64 of CA V actually occupies the same space as the hydroxyl of tyrosine-7 in human CA II if the backbone atoms are superimposed (Fig. 1-3).

A search for other possible ionizable residues within the active site of mouse CA V that may act as proton shuttles identifies four other residues: histidine-7, lysine-91, tyrosine-131, and lysine-132. Histidine-7 is unusual since CA I, II, and III have a conserved tyrosine at this position. The proximity of histidine-7 to the active site and its physiological pKa make it a likely candidate as a proton transfer site. Tyrosine-131, lysine-132, and lysine-91 may also function as proton acceptors, although at a higher pH than histidine.

Inhibition and Physiology of Carbonic Anhydrase V

Following the simultaneous discovery of carbonic anhydrases (Meldrum and Roughton, 1933; Stadie and O'Brien, 1933), much of the early research on carbonic anhydrases focused upon their inhibition by sulfonamide type inhibitors. An understanding of the physiology of carbonic anhydrases has mostly come about from the study of the inhibition of carbonic anhydrases. With the discovery of at least seven distinct mammalian isozymes to date, the differences in inhibition properties of these isozymes have become

increasingly important in trying to determine the specific physiological role of each isozyme.

CA I, II, III, IV, and V have all been found in hepatocytes (Gros and Dodgson, 1988). CA I, II, and III are present in the hepatic cytosol. CA IV is thought to be bound to the plasma membrane. CA V is in the mitochondria of the liver and possibly the kidney (Dodgson *et al.* 1980). Since CA V makes up less than 5% of the total hepatocytic CA activity (Dodgson and Watford, 1990), determining its *in vivo* function has relied upon the effect of sulfonamide inhibitors that could penetrate the membrane of the mitochondria. These studies have been confounded by the presence of the cytosolic CA isozymes.

Early attempts to isolate CA V from guinea pig liver mitochondria (Dodgson *et al.*, 1980; Hewett-Emmett *et al.* 1986) have shown that CA activity in mitochondria is strongly inhibited by sulfonamide type inhibitors. Since acetazolamide reduced the synthesis of citrulline production in isolated liver mitochondria, Dodgson *et al.* (1983) proposed that the function of CA V was to provide bicarbonate for carbamoyl phosphate synthetase in the urea synthetic pathway. Other studies have also suggested that CA V supplies bicarbonate for pyruvate carboxylation and subsequently glucose synthesis (for a review, see Dodgson, 1991b). The inner mitochondrial membrane has a low permeability to bicarbonate and entry of bicarbonate through the general

anion carrier in the inner membrane is also slow (Dodgson *et al.*, 1980; Beavis and Garlid, 1987). Carbonic anhydrase V may be necessary to convert carbon dioxide into a ready supply of bicarbonate inside the mitochondria.

Many sulfonamide and thiazide diuretics also inhibit carbonic anhydrases. These drugs caused a decrease in urea synthesis in perfused rat livers (Häussinger *et al.*, 1986). This led to the hypothesis that the cause of hepatic encephalopathy in patients with liver cirrhosis treated with diuretics is from the inhibition of carbonic anhydrase V, resulting in a decrease in urea synthesis and increased hyperammonemia.

However, none of the above studies has shown that purified carbonic anhydrase V is inhibited by sulfonamides, nor to what extent. One minor aspect of this present work is to determine the K_i of sulfonamide type inhibitors upon purified carbonic anhydrase V.

Enzymatic Mechanism

The uncatalyzed interconversion of CO_2 and HCO_3^- has been extensively studied (Magid and Turbeck, 1968; Liang and Lipscomb, 1988).

The reaction catalyzed by carbonic anhydrases at pH 7 is:



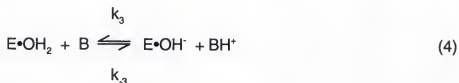
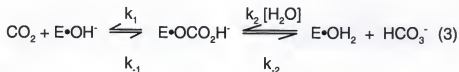
However, at higher pH, where hydroxide concentration is increased, the

following reaction must also be considered:



The uncatalyzed rate constants for reactions (1) and (2) are $3.5 \times 10^{-2} \text{ s}^{-1}$ and $8.5 \times 10^3 \text{ s}^{-1}$ at 25°C respectively. Measurements of a turnover number as great as $1.4 \times 10^6 \text{ s}^{-1}$ have been made for the reaction catalyzed by human carbonic anhydrase isozyme II, making it one of the fastest enzymes known (Khalifah, 1971). The maximal value of k_{cat}/K_M for human CA II is greater than $10^8 \text{ M}^{-1} \text{ s}^{-1}$, approaching a rate value that is diffusion controlled (Khalifah, 1973; Lindskog and Coleman, 1973).

It is generally accepted that the high catalytic rate of interconversion of CO_2 and HCO_3^- occurs through a nucleophilic attack on CO_2 by a zinc-bound hydroxide in the active site of carbonic anhydrases and that the regeneration of this hydroxide requires a separate proton transfer step (Simonsson *et al.*, 1979). Therefore the overall mechanism for the hydration of CO_2 by carbonic anhydrases can be divided into two distinct reactions:



To regenerate the form of the enzyme active in hydration, the proton transfer in equation 4 requires a proton acceptor, B.

The apparent second order rate constant for the reaction in equation 3, k_{cat}/K_M , is equal to $k_1 k_2 / (k_{-1} + k_2)$. Lindskog *et al.* (1984) has shown that k_{cat}/K_M is dominated by k_1 for CA II. The hydration of CO_2 catalyzed by carbonic anhydrase II has a pH profile for k_{cat}/K_M that is described by a titration curve with a single ionization with a pK_a near 7. The ionization of the zinc-bound water is thought to be the determining factor for this apparent pK_a (Simonsson and Lindskog, 1982).

Measurements of carbonic anhydrase II activity at steady state show an inverse relationship between pH and the turnover number, k_{cat} (Pocker and Bjorkquist, 1977). These pH profiles describe a titration curve also with a pK_a of 7, suggesting that the catalytic rate is dependent upon the ionization of a residue, or residues, with pK_a 's near 7.

Site-specific mutagenesis studies have clearly demonstrated that the proton transfer step described by equation 4 is mediated by a single histidine at position 64 of carbonic anhydrase II (Jewel *et al.*, 1991). In CA I there are three possible proton acceptors, His-64, His 67, and His-200 (Lindskog *et al.*, 1984). CA III has been shown to lack significant intramolecular proton transfer except in mutants where a histidine has been inserted at position 64, 65, and

67 (Jewel *et al.*, 1991; Ren *et al.*, 1995). As mentioned previously, carbonic anhydrase V differs substantially from the other isozymes in terms of likely proton shuttle groups. Chapter 4 of this work will present data from several site-specific mutants used to investigate the possible sites of proton transfer in carbonic anhydrase V.

CHAPTER 2 PURIFICATION OF NATIVE CARBONIC ANHYDRASE V

Introduction

The presence of a carbonic anhydrase activity in mitochondria was reported in 1959 by Datta and Shepherd. Over twenty years later a unique mitochondrial specific isozyme of carbonic anhydrase was defined as carbonic anhydrase V (Dodgson *et al.*, 1980). This isozyme has been isolated and amino terminally sequenced from guinea pig and rat liver mitochondria (Hewett-Emmett *et al.*, 1986; Ohlinger, *et al.*, 1993). Comparison of these amino terminal sequences with known carbonic anhydrases confirmed that CA V was indeed a unique isozyme of carbonic anhydrase found in liver mitochondria. A candidate for the complete coding sequence for carbonic anhydrase V was found accidentally by Amor-Gueret and Levi-Strauss (1990) as they were searching a mouse liver library for B2 repeat sequences. The deduced amino acid sequence from this mouse cDNA clone had significant sequence homology, 40-70%, with other carbonic anhydrases, particularly with the partial amino terminal sequences for guinea pig and rat CA V (Figure 2-1).

	-40	-30	-20	-10	1
MUSCA5		MLRRDPRKPL	AILRHVGLLC	ATGPPQRWRFQ	HSCAEHSNC
RCA5-N					
GCA5-N					
RATCA5	MLRAK	MLGRGPYKPL	AILRHMGPLC	ATRPQHWRFO	HSYAEKHSNC
HUMCA5	MLGRNT	WKTSAFSFLV	EQMWAPLWSR	SMRPGRWCSQ	RSCAWQTSNN
	11	21	31	41	51
MUSCA5	ARHPLWTGPV	SSAEGTRQSP	INIQWKDSVY	DPQLAPLRVS	YDAASCRYL
RCA5-N		.VPRGTRQSP	INIQWRN		
GCA5-N		.VPRGTRQSP	INIQRRD?IY	D??LP??KL? Y	
RATCA5	ARHPLWTGPV	SSPGGTQQSP	INIQWTDVY	DPKLAPLRVS	YDAASCRYL
HUMCA5	TLHPLWTVPV	SVPGGTRQSP	INIQWRDSVY	DPQLKPLRVS	YEAASCLYI

Figure 2-1: Sequence comparison of rat (RCA5-N) and guinea pig (GCA5-N) CA V N-termini with the deduced amino acid sequences for mouse (MUSCA5), human (HUMCA5), and rat (RATCA5) CA V. Numbering is based upon sequence alignment with human CA I. Sequences from Ohlinger *et al.*, 1993; Hewett-Emmett, 1986; Ngao, *et al.*, 1993, and Ngao, *et al.*, 1994.

This cDNA sequence was subsequently used as a probe to isolate human and rat CA V cDNA sequences as well (Ngao *et al.*, 1993;1994).

An unusual feature of all three of the cDNA sequences encoding CA V is the addition of 30-40 residues at their amino termini (when aligned and compared with several of the other isozymes of carbonic anhydrase, Figure 1). These additional residues exhibit several of the characteristics of a mitochondrial import signal sequence as discussed in Chapter 1.

The absence of these leader sequences in purified guinea pig and rat CA V proteins supports the hypothesis that they are mitochondrial leader sequences which are proteolytically removed during or after import into the

mitochondria. However, the mature form of CA V found in the mitochondria of mice was not clear from the purification and amino terminal sequencing of guinea pig and rat CA V. Based upon the work of Hewett-Emmett *et al.* (1986) and Ohlinger *et al.* (1993), the mature form of CA V would have an amino terminus that lacked twenty residues found in all of the other carbonic anhydrases. In order to construct a recombinant clone of mouse CA V that closely resembled the mature form of CA V found in the mitochondria, carbonic anhydrase was purified from mouse liver mitochondria and two potential amino terminal sequences were determined for the mature form of the enzyme. Several activity assays for cytosolic and mitochondrial specific enzymes were used to demonstrate that the purified protein was obtained from mitochondria.

Materials And Methods

Preparation of Mouse Liver Mitochondria

Mitochondria were purified from mouse liver homogenates by differential centrifugation using the procedures of Lansman *et al.* (1981) with the addition of a 15 minute incubation with 0.1 % digitonin prior to the final pelleting step. Livers were removed immediately after cervical dislocation of a mouse and placed in ice cold MSB (0.21 M mannitol, 0.07 M sucrose, 0.05 M tris, 0.003 M CaCl_2 , and 0.01 EDTA). A few ml of blood was mixed with

heparin and saved at 4° C for use as a control for contamination by other carbonic anhydrases. Livers were rinsed twice in MSB to remove excess blood. MSB was removed by filtering the livers through a nylon screen.

As much as possible of the following procedures were performed in trays of ice. Centrifuges were maintained at 4° C. Liver tissue was sliced repeatedly with razor blades custom-fitted to an electric carving knife until it had a paste-like consistency. The liver paste was resuspended in 140 ml MSB (less than 0.3 g liver per ml) and passed ten times through a Dounce glass homogenizer with a type A pestle and then ten times through with a type B pestle to disrupt the liver cells. Cellular debris was removed from the homogenized solutions with two low speed spins of 2200 rpm for 5 minutes in a Sorval HB-4 swinging bucket rotor. Pelleted material from the first low speed spin was resuspended in 140 ml MSB, passed through the Dounce glass homogenizer using a type B pestle ten times, and subjected to two additional low speed spins. The supernatants were pooled. Mitochondrial fractions were pelleted by centrifuging the supernatant at 9000 rpm for 30 minutes. Pellets were resuspended in 90 ml of MSB and passed through the glass homogenizer ten times with a type B pestle. The mitochondrial suspension was again centrifuged at 9000 rpm for 30 minutes and pelleted material was resuspended in 90 ml MSB without EDTA. Nine mg of Digitonin (0.1 mg / ml) were added to the suspension. The solution was kept on ice for 15 minutes with occasional stirring. Following the digitonin treatment, the mitochondria

were pelleted again at 9000 rpm for 30 minutes. Following removal of the supernatant, the pellets were weighed, resuspended in a minimum of MSB without EDTA (from 0.3 to 0.5 g mitochondria per ml), quick frozen in a dry ice/ethanol bath, and stored at -70° C.

Purification of Carbonic Anhydrase V from Mouse Liver Mitochondria

Frozen mouse liver mitochondria were thawed, resuspended, and lysed at a concentration of 20 mg protein / ml in a lysis solution containing 1% triton X-100, 100 mM tris, 200 mM sodium sulfate, 1 mM mercaptoethanol at pH 9 supplemented with the following protease inhibitors: 1 mM phenylmethylsulfonylfluoride, 1 mM benzamidine, 5 mM iodoacetamide, 1 mM EDTA, and 2 M leupeptin. Following centrifugation at 23,000 x g for one hour, carbonic anhydrase was purified from the clear supernatant of these mitochondrial lysates by affinity chromatography using a procedure based upon the methods of Khalifah *et al.* (1977). Affinity gel, p-aminomethylbenzenesulfonamide coupled to agarose beads (Sigma Chemicals), was rinsed in a wash solution identical to the lysis solution except for the omission of triton X-100. The washed affinity gel was added to the supernatant of the mitochondrial lysate (1 ml affinity gel per 150 ml mitochondrial lysate) and stirred for 90 minutes at 4° C. The affinity gel was separated from the lysate by 1 minute of low speed centrifugation (1000-2000 x g). Following careful removal of the supernatant, the affinity gel was washed

by resuspending it in 5 volumes of wash solution and then pelleting it again by low speed centrifugation. This batchwise washing step was repeated until absorbance of the supernatant at 260 and 280 nm reached a minimum. The washed affinity gel was loaded onto a column and carbonic anhydrase was eluted with 0.4 M sodium azide, 0.1 M tris, pH 7.1. The collected fractions were checked for eluted protein by looking for an absorbance peak at 280 nm. Protein containing fractions were combined (40 ml total) and dialyzed against 4 l of 15 mM tris, 4 mM mercaptoethanol, pH 8.1. Dialysis buffer was changed every 4 to 8 hours for a total of six buffer changes. The affinity gel eluate was concentrated from a volume of 40 ml down to 1 ml using an Amicon Centriprep concentrator with a 10,000 Da molecular weight cut-off. 0.5 ml of the concentrated eluate was tested for carbonic anhydrase activity by the ^{18}O exchange method described below, and the remainder was further concentrated with an Amicon Centricon concentrator (10,000 Da cut-off) to a volume of 40 μl prior to SDS-PAGE, blotting, and N-terminal analysis.

Total Protein Concentration Assays

Total protein concentrations in liver homogenates and purified mitochondria were determined with the Total Protein Reagent, Sigma Diagnostics kit #541. 20 μl homogenized liver or lysed mitochondria were mixed with 1 ml of Sigma's Total Protein Reagent and incubated at room temperature for 10 minutes. Absorbance was measured at 540 nm and

compared to protein standards prepared from 20, 40, 60, 80, and 100 mg / ml Protein Standard (supplied with Sigma kit # 690 described below) in 1 ml of the Total Protein Reagent.

The concentration of protein in the purified mitochondrial carbonic anhydrase (dialyzed and concentrated affinity gel eluate) was determined using Sigma's Micro Protein Determination Kit #690, a modified Lowry procedure based upon the procedures of Ohnishi and Barr (1978). 200 μ l of the purified carbonic anhydrase were mixed with 2.2 ml of the Biuret Reagent and incubated at room temperature for 10 minutes. 0.1 ml of Folin and Ciocalteu's Phenol Reagent was then added. The mixture was immediately vortexed and then incubated for 30 minutes at room temperature. Absorbance was measured at 725 nm and compared with standard protein concentrations of 0.25, 0.5, 0.75, and 1.0 mg / ml of Sigma's Protein Standard prepared by the same procedure.

Marker Enzyme Assays

Marker enzyme assays were used to determine the purity of isolated mitochondria compared to liver homogenates. Solutions of 1 mg / ml liver homogenate and 1 mg / ml mitochondria were prepared with 1% triton X-100 for use in the following marker enzyme assays.

The activity of glutamate dehydrogenase, a mitochondrial matrix enzyme, was determined using the procedures of Beaufay, *et al.* (1959). 100

μl of 1 mg / ml liver homogenate or 0.1 mg / ml mitochondria were combined with 700 μl of 37.5 mM nicotinamide, 0.4 mM NaCN, 0.125% triton X-100, 1.75 mM NAD, 1.25 mM EDTA, vortexed, and incubated at room temperature for 15 minutes. A Beckman DU600 spectrophotometer was set up to measure absorbance at 340 nm every 10 seconds over a 10 minute time period. The data collection was initiated immediately after addition and complete mixing of 200 μl of 65 mM L-glutamate at pH 7.4 in a 1 ml quartz cuvette. Uncatalyzed reactions rates were determined by substituting water for the enzyme sample and background was determined for each sample by substituting water for the substrate mix of 65 mM L-glutamate. The measured rates were converted to mol NAD reduced $(\text{mg protein})^{-1} \text{ min}^{-1}$ using an extinction coefficient of $1.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for NADH at 340 nm.

Lactate dehydrogenase activity was used as a cytosolic marker enzyme. Lactate dehydrogenase activity was determined using Sigma Diagnostics kit #340 using procedures based on the spectrophotometric method of Wroblewski and LaDue (1955). For every three reactions, 2.7 ml of 0.1 M potassium phosphate buffer, pH 7.5, were added to 0.2 mg NADH (1 substrate vial) and 0.95 ml was aliquotted into eppendorfs. 50 μl of 2% triton X-100 and 15 μl of 1 mg / ml liver homogenate or mitochondria were added and incubated at room temperature for 20 minutes. A Beckman DU600 spectrophotometer was set up to measure absorbance at 340 nm every 5

seconds over a 5 minute time period. The data collection was initiated immediately after addition and complete mixing of the preincubated samples with 35 μ l of 22.7 mM sodium pyruvate, 0.1 M phosphate buffer, pH 7.5 in a 1 ml quartz cuvette. Uncatalyzed reactions rates were determined by substituting water for the enzyme sample and background was determined for each sample by substituting water for the substrate mix of sodium pyruvate. The measured rates were converted to mol NADH oxidized (mg protein)⁻¹ min⁻¹ using an extinction coefficient of $1.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for NADH at 340 nm.

5'-nucleotidase enzyme activity was used as a plasma membrane marker enzyme. Activity was measured using Sigma Diagnostics kit #675 which is based on the methods of Dixon and Purdom (1954). Four different assays were used for each sample to be tested: A) an apparent 5'-nucleotidase phosphatase activity, B) an uncatalyzed apparent 5'-nucleotidase phosphatase activity, C) a nonspecific phosphatase activity, and D) an uncatalyzed nonspecific phosphatase activity. For assay A, 0.96 ml of freshly prepared 2.5 mM adenosine phosphate substrate was mixed with 40 μ l of 1 mg / ml liver homogenate or mitochondria and incubated at 37° C for 2.5 hours. 200 μ l cold 30% TCA was added and reactions were placed on ice for 5-10 minutes. Unconsumed substrate was precipitated by spinning tubes 5 minutes in a microfuge. The supernatant was saved for inorganic phosphorus analysis. Assay B was performed identically except liver homogenate or mitochondria were not added until after the addition of TCA. Assays C and D

were performed identically to assays A and B, respectively, except 0.96 ml of 1.6 mM glycerophosphate, pH 7.5, was used instead of adenosine phosphate. The amount of phosphate released by assay A-D (as determined by the inorganic phosphorus analysis described below) was added or subtracted as follows to determine the pmol phosphate released $\text{mg}^{-1} \text{min}^{-1}$ by 5' nucleotidase activity: (A - B) - (C - D).

Inorganic phosphorus analysis was used to determine the phosphate released during the 5' nucleotidase assays and the glucose-6-phosphatase assays. The analysis was performed using the Phosphorus, Inorganic detection kit #670 from Sigma Diagnostics. This kit employs a modification of the methods described by Fiske and SubbaRow (1925). 800 μl of test sample were mixed with 200 μl of acid molybdate solution (ammonium molybdate $\cdot 4\text{H}_2\text{O}$, 0.125 g / L, in 2.5 N sulfuric acid) and 50 μl of Fiske & SubbaRow reducer (1-amino-2-naphthol-4-sulfonic acid, 0.8%, sodium sulfite and sodium bisulfite). This solution was left at room temperature for 10 minutes and then absorbance at 660 nm was determined. Measurements were compared to a calibration curve based on the results from phosphorus determinations of 5 potassium phosphate standards of 26, 52, 104, and 156 nmol / ml.

Glucose-6-phosphatase activity was used as an endoplasmic reticulum marker enzyme. Activity was measured using the procedure of Swanson (1955). For each assay, 100 μ l 1 mg / ml liver homogenate, mitochondria, or water (blank) was combined with 300 μ l 65 mM maleic acid, pH 6.5 and 100 μ l 0.1 M glucose-6-phosphate. Following an incubation of 15 minutes at 37° C, 200 μ l 30% TCA and 300 μ l water were added. The reactions were centrifuged 10 minutes at 10,000 x g. The resulting supernatant was saved for inorganic phosphorus analysis. The amount of phosphorus detected for the blank was subtracted from the catalyzed reactions resulting in a determination of pmol phosphate released $\text{mg}^{-1} \text{ml}^{-1}$.

Acid phosphatase activity was used as a lysosomal marker enzyme. Activity was measured using the Sigma Diagnostics kit #104: Phosphatases, Alkaline, Acid, Prostatic Acid. For each assay, 100 μ l of 4 mg / ml p-nitrophenol phosphate and 100 μ l of 90 mM citrate, 10 mM chloride, pH 4.8 were mixed and pre-incubated at 37° C for 2 minutes. 40 μ l of 1 mg / ml liver homogenate, mitochondria, or water (blank) were added and the solutions were incubated for 30 minutes at 37° C. The reactions were stopped with the addition of 1 ml 0.1 N NaOH and absorbance at 410 nm was determined after zeroing the spectrophotometer with a blank reaction. The background absorbance of 1.2 ml 0.1 N NaOH mixed with 40 μ l of liver homogenate or

mitochondria was subtracted from the catalyzed measurement. The adjusted absorbances were compared to the absorbances measured for standard solutions of 0.91 to 9.1 μM p-nitrophenol to determine the amount of nmol p-nitrophenol released $\text{mg}^{-1} \text{ min}^{-1}$.

SDS-PAGE and N-Terminal Sequencing

The carbonic anhydrase purified from the mitochondria was separated by electrophoresis on a 10% SDS-polyacrylamide gel, transferred to a Pro-Blot membrane (Applied Biosystems), stained with Coomassie blue, and photographed. The bands at 28, 30, and 31 kDa were sequenced at their N-terminus. Sequence analysis was performed on an Applied Biosystems Automated Gas Phase Sequencer at the Interdisciplinary Center for Biotechnology Research at this University.

Oxygen-18 Exchange Activity Measurements

Carbonic anhydrase activity was determined by measuring the rate of exchange of ^{18}O from $^{12}\text{CO}_2$ to $^{13}\text{CO}_2$ caused by the transitory labeling of the active site of carbonic anhydrase using the method described in Silverman (1982). These exchanges are observed at chemical equilibrium. The reaction solution was in contact with a membrane permeable to gases; CO_2 passing across the membrane entered a mass spectrometer (Extrel EXM-200)

providing a continuous measure of isotopic content of CO_2 . Two rates were determined: R_1 , the rate of interconversion of CO_2 and HCO_3^- ; and $R_{\text{H}_2\text{O}}$, the rate of release from the enzyme of H_2^{18}O (Silverman, 1982). All measurements were performed in 8-10 ml total volume of 100 mM Hepes, pH 7.5, 25 mM total ($\text{HCO}_3^- + \text{CO}_2$), at 10° C. Triton X-100 was added to 1% to ensure lysis of the liver homogenate and mitochondrial samples.

Results

Purification of Mouse Liver Mitochondria

Mitochondria isolated from mouse livers was tested for the presence of several marker enzymes to demonstrate the purity of the mitochondrial preparation. Table 2-1 shows that the activity of glutamate dehydrogenase, a mitochondrial matrix enzyme, was increased 3-fold in the purified liver mitochondria compared to the total liver homogenate. Contaminating cytosol and plasma membrane, as indicated by the presence of lactate dehydrogenase and 5' nucleotidase, were reduced to less than 11% and 5%, respectively. The amounts of lysosomal and endoplasmic reticulum components that copurified with the mitochondria were somewhat higher with 27% and 21% of the original activity of acid phosphatase and glucose-6-phosphatase still present.

Table 2-1
Marker Enzyme Analysis of Mitochondria Preparation

	Glutamate Dehydrogenase (Mit Matrix)	Lactate Dehydrogenase (Cytosol)	5' Nucleotidase (Plasma Membrane)	Acid Phosphatase (Lysosomes)	Glucose-6- Phosphatase (Endoplasmic Reticulum)
Liver Homogenate	5.3 ± 0.3	220 ± 21	4.4 ± 0.5	146 ± 5	12 ± 4
Liver Mitochondria	14.8 ± 0.2	25 ± 5	0.22 ± 0.09	39 ± 20	2.5 ± .5
RSA	2.8	0.11	.05	.27	.21

Enzyme activities are expressed as nmol NAD reduced $\text{mg}^{-1} \text{min}^{-1}$ for Glutamate Dehydrogenase and Lactate Dehydrogenase. 5' Nucleotidase activity and Glucose-6-phosphatase activity is expressed as pmol phosphate released $\text{mg}^{-1} \text{min}^{-1}$. Acid Phosphatase activity is expressed as nmol p-nitrophenol released $\text{mg}^{-1} \text{min}^{-1}$. Relative specific activity (RSA) is the ratio of mitochondrial specific activity / liver homogenate specific activity.

Table 2-2
 R_1 and R_{120} for Mouse Mitochondria and Mouse Blood in the Presence and Absence of Cupric Ions.

Sample	R_1 (mol sec^{-1})	R_{120} (mol sec^{-1})
Mitochondria	0.18 ± 0.02	0.42 ± 0.09
Blood	0.11 ± 0.03	0.27 ± 0.08
Mitochondria + Cu^{2+}	0.16 ± 0.01	0.39 ± 0.04
Blood + Cu^{2+}	0.06 ± 0.01	0.01 ± 0.01
Mitochondria + Blood	0.28	0.83
Mitochondria + Blood + Cu^{2+}	0.25	0.65

Data were measured in 8 ml of 100 mM Hepes, 1% triton X-100, at pH 7.4 and 10° C. The total concentration of $\text{CO}_2 + \text{HCO}_3^-$ was 25 mM. The same quantities of mitochondria, blood, and Cu^{2+} were used for each individual assay as well in the mixtures. Mitochondria were tested at 6.9 mg mitochondria / ml, whole mouse blood at a dilution of 1:533, and Cu^{2+} at 10 mM.

Table 2-3
Purification of Mitochondrial Carbonic Anhydrase

Purification Step	Total Protein (grams)	Total Activity (mol sec^{-1})	Specific Activity (mol $\text{sec}^{-1} \text{mg protein}^{-1}$)	Yield (%)	Purification (fold)
Liver Homogenate	20.4	1760	0.0865	100	6
Liver Mitochondria	6.1	88	0.0145	5	1
Affinity Gel Eluate	0.00015	42	281	2.4	19400

R_1 was used as a measure of activity. Data were measured in 100 mM Hepes, 1% triton X-100, at pH 7.4 and 10° C. The total concentration of $\text{CO}_2 + \text{HCO}_3^-$ was 25 mM.

Identification of Carbonic Anhydrase Activity in Mouse Liver Mitochondria

Carbonic anhydrase activity was found in the purified mouse mitochondria using the ^{18}O exchange method. The rates of interconversion of CO_2 and HCO_3^- at chemical equilibrium, R_1 , and the rates of dissociation of water from the active site, $R_{\text{H}_2\text{O}}$, catalyzed by various forms of carbonic anhydrase in samples of mitochondria and blood are shown in Table 2-2. The values of R_1 and $R_{\text{H}_2\text{O}}$ for the mitochondrial sample indicate a low, but significant, amount of carbonic anhydrase activity exists in isolated mitochondria as compared to mouse blood. Cupric ions, inhibitors of $R_{\text{H}_2\text{O}}$ in CA II (Tu *et al.*, 1981), eliminated almost all $R_{\text{H}_2\text{O}}$ carbonic anhydrase activity in mouse blood, but had little effect upon the mitochondrial carbonic anhydrase activity. The inhibition of the blood component of a mitochondrial lysate + blood + cupric ion mixture demonstrated that the lack of effect of the cupric ions on the mitochondrial CA activity was not caused by sequestration of a component of the lysed mitochondrial suspension.

1 μM ethoxzolamide, a potent inhibitor of CA I, II, and IV, was also tested for its effects upon CA activity. The addition of ethoxzolamide completely abolished almost all CA activity in every sample of mitochondria or blood tested (data not shown).

Purification of Mitochondrial Carbonic Anhydrase

Table 2-3 shows the amount of carbonic anhydrase activity recovered from the liver mitochondria after affinity chromatography. The specific activity of carbonic anhydrase present in the purified sample was increased over 19,000-fold compared to the activity in the mitochondria. Almost half of all of the carbonic anhydrase activity detected in the mitochondria preparation was recovered during the purification. The six-fold decrease in activity and yield of carbonic anhydrase following the purification of the mitochondria from the liver homogenate was expected since several cytosolic and blood carbonic anhydrases were removed during this step.

N-Terminal Analysis of Native Carbonic Anhydrase V

The proteins present in the preparation of mitochondrial carbonic anhydrase are shown on a Coomassie blue stained blot of a 10% SDS-polyacrylamide gel in Figure 2-2. The bands at 28, 30, and 31 kDa were excised from the blot and sequenced at their N-termini. The bands at 30 and 31 kDa were mixtures of proteins which appeared to contain the sequence QHS, but the sequencing data was not definitive. Figure 2-1 shows this sequence beginning at position -1 of the deduced amino acid sequence from the cDNA for mouse CA V. The band at 28 kDa showed one major component

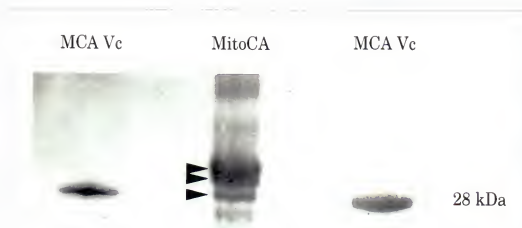


Figure 2-2. Stained blot of SDS-polyacrylamide gel electrophoretic separation of proteins from the purification of mitochondrial carbonic anhydrase, MitoCA. Lanes marked with MCA Vc are the truncated recombinant form of MCA V described in Chapter 3.

which had an N-terminal sequence of AEGTR. This sequence is found at positions 23 through 27 in the mouse CA V sequence shown in Figure 2-1. These results indicated that the protein isolated from the mitochondria matched an internal portion of the deduced amino acid sequence of the carbonic anhydrase cDNA discovered by Amor-Gueret and Levi-Strauss (1990). The 28 kDa protein had an amino terminus consistent with the removal of 52 residues from the full length CA V encoded by the cDNA. The data from the N-terminal analysis of the 30 and 31 kDa bands also suggested that CA V from mitochondria may have an amino terminus that begins at position -1 after the removal of only 29 residues. Based on the deduced amino acid sequence of Amor-Gueret and Levi Strauss (1990), the predicted molecular weights for truncated forms of mouse CA V lacking 29 or 52 residues would be 28 or 31 kDa.

Discussion

Carbonic anhydrase purified from mouse liver mitochondria has an amino terminal sequence that confirms the identity of the cDNA of Amor-Gueret and Levi-Strauss (1990) to be carbonic anhydrase V. The clearest amino terminal sequence of CA V that I found in lysates of liver mitochondria that were heavily inhibited with protease blockers was a

truncated form that lacks a region homologous to the initial 22 or 23 residues found in carbonic anhydrases I, II, and III. This truncated form of CA V is within one residue of the homologous amino terminal sequence positions determined for CA V isolated from rat and guinea pig mitochondria as shown in Figure 2-1. However, it is still quite possible that this short form is a proteolytic product of a full length CA V found in the mitochondrial matrix.

Ngao *et al.* (1993; 1994) have expressed human, rat, and mouse CA V in COS cells and found amino termini corresponding to position -1 for mouse and rat CA V and position 3 for human CA V. However, these overexpressed CA V proteins were purified from whole cell lysates of COS cells, so the degree of mitochondrial processing is uncertain.

All of the results to date indicate that a portion of the carbonic anhydrase V amino terminal sequence is absent in samples isolated from mitochondria. This is consistent with many other mitochondrial matrix proteins which have an amino terminal leader sequence that is proteolytically removed during import into the mitochondria (Hartl *et al.*, 1989). The amino terminus of mouse CA V has several of the structural properties of the mitochondrial leader sequences described by Hartl *et al.* (1989). It is rich in positively charged residues, lacks acidic residues, and could possibly form an amphipathic helix.

Arginine 12, proline 14, and threonine 17 comprise a partially conserved three amino acid motif (arginine at -10, hydrophobic residue at -8, and serine, threonine, or glycine at position -5 relative to the cleavage point) found in mitochondrial leader peptides that undergo a two-step cleavage during import (Hendrick *et al.*, 1989). This motif predicts a cleavage after residue 21 of mouse CA V, resulting in an amino terminus with an initial sequence of SAEGT that is within one residue of the results of my purification and sequencing of mouse CA V and in direct alignment with the results for guinea pig and rat CA V (Hewett-Emmett *et al.*, 1986; Ohlinger *et al.*, 1993).

The mature form of mouse CA V was not unambiguously identified by these studies. Indeed, there is substantial evidence to indicate that mouse CA V could have an amino terminus that begins near position 1, position 22, or both. It would be interesting to see if the mouse CA V cDNA transfected into a mouse cell line will be processed similar to the results seen by transfecting mouse CA V into human COS cells by Ngao *et al.* (1994).

There is little sequence conservation in the region of residues 1-22 of CA V when compared to other isozymes (see Figure 1, Chapter 1). CA I, II, and III have nine highly conserved residues within this region. However, the only two residues of CA V that are even partially conserved in this region, serine 8 and tryptophan 16, match two structurally important residues in CA II

that comprise the internal portion of a loose alpha helix in the crystal structure of CA II (Eriksson *et al.*, 1988). Therefore, evolutionary sequence comparisons provide some support for both the presence and the absence of residues 1-22 in the mature form of mouse CA V.

The mitochondrial CA preparation could have been contaminated by several other isozymes, notably CA I, II, III, and IV. The possibility of contamination by CA III, a sulfonamide resistant isozyme, could be eliminated since all mitochondrial carbonic anhydrase activity was completely inhibited by ethoxzolamide inhibition. The lack of inhibition by cupric ions on the carbonic anhydrase activity detected in the mitochondria demonstrated that CA I and CA II were not present as well. Tu *et al.* (1981) have shown that $\mu\text{M Cu}^{2+}$ inhibits the rate of dissociation of water from the active site, $R_{\text{H}_2\text{O}^+}$ of CA II, most likely by binding to histidine 64 and interfering with proton transfer. Since the proton transfer step is thought to be mediated by a histidine in CA I (Lindskog *et al.*, 1984), it is not surprising that Cu^{2+} inhibited almost all of the $R_{\text{H}_2\text{O}}$ CA activity in the mouse blood sample. Although there are no reports of inhibition of CA IV by cupric ions, it is likely that the histidine at position 64 of this isozyme is similarly affected. The lack of inhibition of CA V by cupric ions points that this isozyme may function differently than the other isozymes, especially in terms of proton transfer.

CHAPTER 3 CLONING, SEQUENCING, MUTAGENESIS, EXPRESSION, AND PURIFICATION OF CARBONIC ANHYDRASE V

Introduction

Amor-Gueret and Levi-Strauss (1990), while screening a mouse liver cDNA library for mRNA clones containing a mouse B2 repeat, isolated a cDNA encoding a protein with strong sequence similarities to a carbonic anhydrase. Of seven tissues tested, messenger RNA was detectable only in liver (Amor-Gueret and Levi-Strauss, 1990). Examination of this cDNA sequence and comparison with the partial sequence of Hewett-Emmett *et al.* (1986) suggested that the cDNA encoded the mouse homolog of guinea pig mitochondrial carbonic anhydrase (CA V).

Chapter 2 described the purification of carbonic anhydrase from mouse liver mitochondria and the use of NH₂-terminal sequence analysis to confirm that this cDNA encoded mouse CA V. The NH₂-terminal sequencing results also suggested that mouse CA V may exist as two different mature forms within the mitochondria.

The cDNA sequence of Amor-Gueret and Levi-Strauss (1990) was used to amplify and clone a full-length CA V sequence from mouse liver RNA

by reverse transcription-PCR (Heck *et al.*, 1994). The entire mouse CA V coding sequence (MCA Va) was expressed using the T7 expression vector system described in Tanhauser *et al.* (1992). Two shorter forms of the enzyme, MCA Vb and MCA Vc were also expressed. These shorter forms were truncated at the amino terminus to correspond with the NH₂-termini of CA V purified from mouse liver mitochondria. All three forms of the expressed proteins were purified and characterized by NH₂-terminal analysis.

Two independently obtained clones of the coding sequence of mouse CA V were sequenced to confirm the published sequence of Amor-Gueret and Levi-Strauss and to identify any sequence errors that may have been introduced during cloning.

Several site specific mutants of carbonic anhydrase V were constructed to investigate the function of three unique residues, tyrosine 64, phenylalanine 65, and tyrosine 131, found in the active site of CA V. All of these mutant constructs expressed as active carbonic anhydrase enzymes and DNA sequencing confirmed that the coding sequence was indeed changed as expected.

Materials and Methods

Cloning of Mouse CA V

The cloning of the full length mouse CA V coding sequence was done by Dr. Susan Tanhauser, in the laboratory of Dr. Philip Laipis at the University of Florida. The cloning of the two NH₂-terminal deletion mutants, MCA Vb and MCA Vc, was done by Ms. Rajyalakshmi Manda in the same laboratory. The mouse CA V coding sequence was reverse-transcribed from BALB/c mouse liver RNA using an antisense 3' -end primer (nucleotides 1030-1049; a *Bam* HI site was added to the 5'-end) based upon the cDNA sequence of Amor-Gueret and Levi-Strauss (1990). The products of first strand synthesis were amplified using the polymerase chain reaction and a 5'-sense primer (nucleotides 105-123; an *Eco* RI site was added to the 5'-end). A major PCR product of 962 base pairs was obtained. It was inserted into a Bluescribe vector (Stratagene) that had been cut with *Sma* I and T-tailed (Marchuk *et al.*, 1991). The cloned products were identified as mouse CA V by restriction site analysis and partial DNA sequencing. A full-length coding sequence, CA Va, as well as two deletion mutants, mouse CA Vb and CA Vc, were synthesized by PCR from the initial PCR-derived clones using the original 3'-oligonucleotide and three new 5'-primers, which added a *Nde* I

	-30	-20	-10	1	11	21
			↑	↑		
Mouse CA Va	MLRRDPRKPL	AILRHVGLLC	ATGPQRWRFQ	HSCAEHSNC	ARHPLWTGPV	SSAEGTRQSP
Mouse CA Vb			MQ	HSCAEHSNC	ARHPLWTGPV	SSAEGTRQSP
Mouse CA Vc						..SAEGTRQSP

Figure 3-1. NH₂-terminal amino acid sequence of mouse CA V. The numbering is based upon the sequence for CA I. The sequence for CA Va is the deduced amino acid sequence from the nucleotide sequence of Amor-Gueret and Levi-Strauss (1990). CA Vb and CA Vc are truncated proteins which correspond to the mature forms of CA V found in the mitochondria of mouse livers. The presence of a methionine at the initial position of recombinant CA Vb and the absence of a methionine for CA Vc were detected by amino terminal analysis. The *daggers* designate the most abundant amino-terminal residues found in several preparations of CA Va.

restriction site and Met start codon at the positions shown in Figure 3-1. The *Nde* I and *Bam* HI restriction sites allowed the constructs to be inserted into the pET31 T7 expression vector (Tanhauser *et al.*, 1992). These clones were transformed into *Escherichia coli* BL21 (DE3) pLysS, a strain optimized for T7 RNA polymerase expression.

Sequencing of the Mouse CA V Coding Sequence

The entire CA V coding sequence cloned into Bluescribe (Stratagene) was sequenced as well as the CA V regions of the pET31 expression vectors (Tanhauser *et al.*, 1992) to confirm the published sequence of Amor-Gueret and Levi-Strauss and to identify any sequence errors that may have occurred during cloning.

Plasmid DNA was prepared for sequencing by an alkaline lysis miniprep procedure, followed by an additional PEG precipitation to remove RNA. This alkaline lysis miniprep procedure was also used extensively to prepare DNA samples for restriction analysis during mutagenesis and cloning procedures. 4 ml of LB media (Luria broth: 5 g yeast extract, 5 g NaCl, 10 g bactotryptone, pH 7.4, per liter of deionized water) containing 100 mg / l ampicillin were inoculated with TG1 or BL21 cells containing the plasmid to be sequenced. The cultures were grown overnight at 37° C to saturation. Cells were pelleted in a 1.5 ml eppendorf tube by a 20 second centrifugation followed by removal of the supernatant. The pellets were resuspended by vortexing in 100 µl of 50 mM glucose, 10 mM EDTA, 25 mM tris-HCL, pH 8.0. After a 5 minute incubation at room temperature, 200 µl of freshly prepared 0.2 N NaOH, 1% (w/v) SDS was added and mixed by gentle rocking. Solutions were placed on ice for at least 5 minutes. The solution was neutralized with 150 µl of ice cold 5 M potassium acetate, pH 4.8, and vortexed on high for at least 2 seconds. Following a 5 minute incubation on ice, the samples were centrifuged for 1 minute and the supernatant was transferred to a clean microfuge tube. Samples were ethanol precipitated by adding 0.9 ml of cold ethanol and centrifuging for 10 minutes at 4° C. After removing the supernatant, the pellets were washed with 0.9 ml cold 70% ethanol and centrifuged for 1 minute.

Following removal of the supernatant, the pellets were dried under vacuum for 15 minutes. The pellets were dissolved in 400 μ l 10 mM tris-HCL, 1 mM EDTA, pH 8 (TE) and 16 μ l 1 mg / ml boiled RNase A. The solutions were incubated for 30 minutes at room temperature and then protein was extracted with an equal volume of phenol:chloroform (equal parts v/v). The aqueous phase was collected and 3 M sodium acetate, pH 5.2, was added to bring the solution to 0.3 M sodium acetate. DNA was ethanol precipitated with the addition of 0.9 ml cold ethanol and centrifugation for 10 minutes at 4° C. Pellets were washed with 70% cold ethanol and then again dried under vacuum. Dried pellets were dissolved in 50 μ l TE. DNA was precipitated again by adding 30 μ l of 20% polyethylene glycol (PEG), MW 8000, 2.5 M NaCl. Solutions were left on ice for 1 hour and then centrifuged for 15 minutes at 4° C. The pellets were washed with cold 70% ethanol, dried under vacuum, and dissolved in 20 μ l TE.

Sequencing of double stranded DNA plasmids was by cycle sequencing using the procedures and materials in the Promega fmol Cycle Sequencing kit. Table 3-1 includes the oligonucleotides used as sequencing primers.

Table 3-1

Oligonucleotides Used for the Cloning, Sequencing, and Mutagenesis of Mouse CA V

Name	Sequence (5'-3')	Description
DS44	<u>CCG AAT TCA TAT</u> GCT CAG GAG AGA CCC CCG C	101-132. NH ₂ -end with added Nde I and Eco RI sites.
DS45	<u>CGG ATC</u> CTC AGA TTA AGA CCT CAT CTT G	M1011-993. COOH-end with added Bam HI site
DS58	<u>CGC GGA</u> TCC GGT CTG CTC TGC CTA TC	M1049-1030. COOH-end with added Bam HI site.
DS 59	<u>CGG AAT TCA</u> GCC AAG ATG CTC AGG AGA	105-133. MCA Va NH ₂ -end with added Eco RI site.
DS100	<u>CAT ATG</u> TCA GCA GAA GGC ACC CG	265-281. MCA Vc NH ₂ -end with added Nde I site.
DS101	<u>CAT ATG</u> TCA GCA GAA GGC ACC CG	198-215. MCA Vb NH ₂ -end with added Nde I site.
DS102	CAC TGG <u>CQA</u> CTT CTT CCA GG	384-403. Tyr 64 to His 64. Creates a Bal I site.
DS103	CAC TGG <u>CGC</u> <u>TTT</u> CTT CCA GG	384-403. Tyr 64 to Ala 64. Creates a Hae II site.
DS146	TGC TCT GAG CCC CAT TCA TC	M521-502. Exon 3 minus primer.
DS150	GAA <u>CTC</u> CAC GAA ATA TGA AAA <u>TCA</u> CAA	567-593. Tyr 131 to His 131. Knocks out an Eco RI site.
DS151	GAA <u>CTC</u> CAC GAA ATA TGA AAA <u>TGC</u> CAA GA	567-595. Tyr 131 to Ala 131. Knocks out an Eco RI site
DS159	GGG GAG CAA CAG ATG AAT	491-508. Exon 3 plus primer.
DS160	AGC CAG TGT TCC AGA GGT	M391-374. Exon 2 minus primer.
DS161	GCC AAG ATG CTC AGG AGA	106-123. NH ₂ -end primer
DS162	CCA GGG TAG GTC CAG TAA	M785-768. Exon 6 minus primer.
DS163	GGT CAT CGG CGT GTT TCT	624-641. Exon 4 plus primer.
DS164	CGG TCT GCT CTG CCT ATC	M1047-1030. COOH-end primer
DS165	CTC ACC ACC CCG CCA CTT	790-807. Exon 6 plus primer
PL97	CAG AAA CAG GCC GAT GAC	M642-625. Exon 4 minus primer

Numerical locations are based on the sequence of Amor-Gueret and Levi-Strauss (1990). An M prior to the numerical position indicates that the oligonucleotide sequence is based on the complementary sequence. Nucleotides not found in the published sequence are underlined.

Each sequencing primer was end-labeled with [γ - ^{32} P] adenosine triphosphate (ATP) using T4 polynucleotide kinase. 2 μ l of template DNA (usually approximately 1 μ g was used although Promega claimed fmol amounts could be used) were mixed with the end labeled primers and TAQ DNA polymerase in the supplied sequencing buffer. 2 μ l of this primer / template mix were added to four 0.5 ml thermal cycler tubes preloaded with 4 μ l of dNTPs + ddATP, dNTPs + ddCTP, dNTPs + ddGTP, or dNTPs + ddTTP. Sequencing reactions were overlaid with 20 μ l mineral oil and placed in a thermal cycler programmed to heat the samples to 95° C for 2 minutes followed by 30 cycles of 95° C for 30 seconds, 42° C for 30 seconds, and 70° C for 1 minute. At the end of the thermal cycling, 3 μ l of Promega stop buffer were added and reactions were placed at -20° C until ready for loading. Just prior to loading on a 6% polyacrylamide, 6M urea sequencing gel, samples were heated to 72° C for 2 minutes. Following electrophoresis, gels were transferred to Whatman 3M paper, covered with plastic wrap, and visualized by autoradiography on Kodak X-Omat XAR film.

Mutagenesis of CA V

Mutant carbonic anhydrase V enzymes were created using the methods of Kunkel *et al.* (1987). No subcloning of the pET31 CA V vectors into M13

was necessary since Tanhauser *et al.* (1992) engineered an f1 replication origin for the efficient synthesis of the single stranded DNA (ssDNA) template needed for this mutagenesis procedure. Single stranded pET31 CA V DNA was produced by transforming a pET31 CA V vector into *E. coli* BW313 (a *dut*⁻, *ung*⁻ strain which misincorporates uracils in place of thymines) and inducing ssDNA synthesis by incubating these cells in presence of VCS-M13 helper phage (Stratagene). Following the preparation of the ssDNA, a phosphorylated mutagenic oligonucleotide (see Table 3-1 for oligos used) was annealed to the single stranded template and used to prime second strand synthesis in the presence of T4 DNA polymerase, T4 DNA ligase, ATP, and dNTPs. The resulting hybrid double stranded DNA plasmid, consisting of the template uridine-containing DNA strand and a mutated strand without uridines, was transformed into *E. coli* strain TG1 and plated onto LB agar plates supplemented with 100 µg / ml ampicillin to screen for cells containing the ampicillin resistant pET31 plasmid. Since TG1 preferentially degrades uracyl-containing DNA, the yield of mutated plasmids found is enhanced. Each mutagenic oligonucleotide was designed to alter a restriction site. Mutant plasmids were detected by restriction digests of DNA purified using the miniprep procedure described above. Every mutant plasmid used for

subsequent expression of mutant CA V was DNA sequenced to confirm the altered sequence introduced.

Expression of Mouse CA V in *E. Coli*

The growth of *E. coli* BL21 (DE3) pLysS cells containing mouse CA V constructs and the subsequent expression of CA V protein was optimized for maximum levels of expressed CA V protein. The most effective growth conditions were found to include a lengthening of the incubation times described in Tanhauser *et al.* (1992) as described below. 4 ml of growth media (LB supplemented with 200 mg / l ampicillin and 35 mg / l chloramphenicol) were inoculated with *E. coli* BL21 (DE3) pLysS cells containing a pET31 mouse CA V construct and incubated at 37° C for 8-12 hours. Fresh growth media was inoculated with these cultures at a ratio of 1000 to 1 of fresh media to culture, and incubated at 37° C with orbital shaking at 260 rpm. The optical density (OD) of the culture was monitored at 600 nm. Upon reaching an OD₆₀₀ of 1, 50 µM IPTG, 10 µM ZnSO₄, and 0.2% (w/v) glucose were added. The cells were incubated an additional 4 to 6 hours after induction. Cells were harvested by centrifugation at 6000 x g for 10 minutes and stored frozen at -70° C.

Purification of Expressed Mouse CA V

The purification procedure for CA V is based upon the methods used to purify carbonic anhydrase II as described by Khalifah *et al.* (1977). Frozen cell pellets containing expressed CA V were thawed and resuspended in 1 ml cold wash buffer per liter of original culture. Wash buffer consists of 100 mM tris, 200 mM sodium sulfate, pH 9 with the addition of the following protease inhibitors for the purification of the full length MCA Va expression product: 1 mM phenylmethylsulfonylfluoride, 1 mM benzamidine, 5 mM iodoacetamide, 1 mM EDTA, and 2 M leupeptin. DNase I was added at 1 mg per 10 ml of suspended cells and solutions were stirred for 1 hour at 4° C. Following centrifugation at 23,000 x g for one hour, carbonic anhydrase was purified from the clear supernatant of these bacterial lysates by affinity chromatography using a procedure based upon the methods of Khalifah *et al.* (1977). Affinity gel, p-aminomethylbenzenesulfonamide coupled to agarose beads (Sigma Chemicals), was rinsed in the wash buffer. The washed affinity gel was added to the supernatant (1 ml affinity gel per 20 ml supernatant) and stirred for 90 minutes at 4° C. The affinity gel was separated from the bacterial lysate by 1 minute of low speed centrifugation (1000-2000 x g). Following careful removal of the supernatant, the affinity gel was washed by resuspending it in 5 volumes of wash solution and then pelleting it again by

low speed centrifugation. This batchwise washing step was repeated until absorbance of the supernatant at 260 and 280 nm reached a minimum. The washed affinity gel was loaded onto a column and carbonic anhydrase was eluted with 0.4 M sodium azide, 0.1 M tris, pH 7.1. The collected fractions were checked for eluted protein by looking for an absorbance peak at 280 nm. Protein containing fractions were combined and dialyzed against 4-6 l of 15 mM tris, 4 mM mercaptoethanol, pH 8.1. Dialysis buffer was changed every 4 to 8 hours for a total of six buffer changes. Most preparations of CA V were stored frozen at -20° C in small aliquots although some samples were kept at 4° C for testing that occurred within a day of the purification.

The purity and molecular weight of each purified enzyme was checked by electrophoresis on a SDS-polyacrylamide gel as shown in Figure 3-2. The expressed enzymes, CA Va, Vb, and Vc were also separated by SDS-polyacrylamide gel electrophoresis, blotted onto a Pro-Blot membrane (Applied Biosystems), and amino terminally sequenced on an Applied Biosystems Automated Gas-phase Sequencer (Model 473A) at the Interdisciplinary Center for Biotechnology Research at this University.

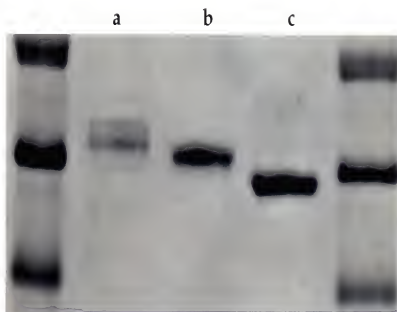


Figure 3-2. Coomassie Blue-stained electrophoretic separation of purified recombinant CA V enzymes on a SDS-polyacrylamide gel. Lane A, mouse CA Va; lane B, mouse CA Vb; lane c, mouse CA Vc. The outer lanes are molecular weight standards (Amersham Rainbow protein molecular weight markers, RPN.755).

Results

Cloning of Mouse CA V

Reverse transcription of BALB/c liver mRNA followed by PCR amplification yielded several independent clones very similar to the cDNA reported by Amor-Gueret and Levi-Strauss (1990). The sequences coding for the three forms of mouse CA V were inserted into the T7 expression vector pET31 (Tanhauser *et al.*, 1992). Their amino termini are shown in Figure 3-1. CA Va contains the full length coding sequence including the 29 amino acids at the NH₂-terminus which could represent a mitochondrial targeting sequence. This amino terminal sequence has no counterpart in CA I, II, or III. CA Vb and CA Vc are shorter forms of CA V representing two possible mature forms of found in the mitochondria.

Two independently obtained clones of CA V were sequenced to check for cloning errors and to check for possible sequence variations due to strain differences; Amor-Gueret and Levi-Strauss (1990) obtained their cDNA from B10.HTT mice while the clones used in this study originated from the cDNA of BALB/c mice. The full sequence for mouse CA V from BALB/c mice is listed in Figure 3-3. The underlined sequences represent changes from the originally published sequence of Amor-Gueret and Levi-Strauss (1990). There were 8

1
 ATGCTCAGGAGAGACCCCGCAAGCCCTTAGCCATCCTCAGGCATGTGGGACTTCTCTGT
 M L R R D P R K P L A I L R H V G L L C
 61
 GCCACAGGGCCACAGCGCTGGCGTTTCCAGCATTCCTGTGCAGAGGAACACAGCAACTGT
 A T G P Q R W R F Q H S C A E E H S N C
 121
 GCCCGACACCCCTCTGGACTGGCCAGTGTCTCAGCAGAAGGCACCCGGCAGTCTCCC
 A R H P L W T G P V S S A E G T R Q S P
 181
 ATCAACATCCAGTGAAGGACAGTGTGTATGACCCGAGCTGGCACCCTCAGGGTCTCC
 I N I Q W K D S V Y D P Q L A P L R V S
 241
 TATGATGCCGCGTCTGCAGATACCTCTGGAACACTGGCTACTTCTTCCAGGTGGAAGTTT
 Y D A A S C R Y L W N T G Y F F Q V E F
 301
 GACGATTCCTGTGAGGATTACAGGGATCAGCGGTGGGCCCTCTGGGAAACCACTACAGGCTG
 D D S C E D S G I S G G P L G N H Y R L
 361
 AAGCAGTTTCATTTCCTGAGGAGCAACAGATGAATGGGGCTCAGAGCAGCAGTGGAC
 K Q F H F H W G A T D E W G S E H A V D
 421
 GGCCATACCTACCCAGCTGAGCTCCATCTGGTTCACTGGAATTCACGAAATATGAAAAAT
 G H T Y P A E L H L V H W N S T K Y E N
 481
 TACAAGAAAGCCTCCGTGGGGGAGAACGGGCTGGCGGTATCGGCGTGTCTTCTGAAGCTC
 Y K K A S S V G E N G L A V I G V F L K L
 541
 (C)
 GGGGCGCATCACCAGGCTCTGCAGAAGCTGGTGGATGTCTTGGCCGAAGTAAGACACAAG
 G A H H Q A L Q K L V D V L P E V R H K
 601
 (C)
 GATACACAGGTGGCCATGGGACCCTTTGACCCCTCTTGTCTGATGCCTGCGCTGCCGGGAT
 D T Q V A M G P F D P S C L M P A C R D
 661
 TACTGGACCTACCTGGCTCCCTCACCACCCCGCCACTTGCTGAGTCAGTCACCTGGATT
 Y W T Y P G S L T T P P L A E S V T W I
 721
 (C) (G)
 GTGCAGAAGACGCTGTGAGGTGTCCCCGAGCCAGCTATCCATGTTCCGCACACTCTTG
 V Q K T P V E V S P S Q L S M F R T L L
 781
 TTCTCCGGCGAGGTGAGGAAGAGGATGTGATGGTGAACAACCTACCGCCCGCTCCAGCCC
 F S G R G E E E D V M V N N Y R P L Q P
 841
 (C) 901
 CTCAGGGACCGCAAACCTTCGCTCGTCTTCCGGCTTGATCGGACCAAGATGAGGTCT
 L R D R K L R S S F R L D R T K M R S

Figure 3-3. Nucleotide and deduced amino acid sequence for the coding region of CA V cDNA from BALB/c mice. The full length sequence, including the putative mitochondrial leader sequence, is shown. Differences from the sequence of Amor-Gueret and Levi-Strauss (1990) are underlined. Parenthesis enclose the nucleotides reported by Amor-Gueret and Levi-Strauss (1990). Asterisks mark inserted nucleotides that were not in the originally published sequence.

sequence changes out of the 900 nucleotides that compose the coding region for mouse CA V. Three of those changes, at positions 558, 771, and 867, are silent and do not change the amino acid sequence. However, the other 5 changes do result in changes to the coding sequence for mouse CA V. The changes at positions 643 and 764 change the codons for a leucine and a threonine into methionine codons. An even more significant change was caused by the insertion of three nucleotides at positions 451, 456, and 459. These insertions resulted in two sequential amino acids, phenylalanine-methionine, being changed to three amino acids, valine-histidine-tryptophan. This restored a conserved amino acid sequence found in all but one of the carbonic anhydrase isozymes shown in Figure 1-1.

Sequencing of the pET expression vectors encoding the various forms of CA V used in this study showed that all of the sequences were correct and in the correct reading frame.

Mutagenesis of Mouse CA V

Table 3-2 lists all of the vectors used to express the full length and truncated forms of wild type mouse carbonic anhydrase V and its mutants.

The following plasmids were created by site-specific mutagenesis of pET31f1mMCA5: pET31f1mMCA5Y64A, pET31f1mMCA5Y64H, and

Table 3-2

Expression Vectors of CA V and CA V Mutants

Vector Name	Protein Product
pET31f1mMCA5	Mouse CA Va
pET31f1mMCA5b	Mouse CA Vb
pET31f1mMCA5c	Mouse CA Vc
pET31f1mMCA5Y64A	Mouse CA Va Y64A
pET31f1mMCA5cY64A	Mouse CA Vc Y64A
pET31f1mMCA5Y64H	Mouse CA Va Y64H
pET31f1mMCA5cY64H	Mouse CA Vc Y64H
pET31f1mMCA5cY131A	Mouse CA Vc Y131A
pET31f1mMCA5cY131H	Mouse CA Vc Y131H
pET31f1mMCA5cY64A/Y131A	Mouse CA Vc Y64A/Y131A
pET31f1mMCA5Y64H/F65A	Mouse CA Va Y64H/F65A
pET31f1mMCA5cY64H/F65A	Mouse CA Vc Y64H/F65A

pET31f1mMCA5Y64H/F65A. The pET31f1mMCA5c vector was mutated by site specific mutagenesis to create pET31f1mMCA5cY131H and pET31f1mMCA5cY131A. The mutant plasmids, pET31f1mMCA5cY64H, pET31f1mMCA5cY64A, and pET31f1mMCA5cY64H/F65A were created by ligating a 301 base pair (bp) Pst I restriction fragment containing the mutated sequence into a backbone derived from pET31f1mMCA5c that had the 301 bp Pst I fragment removed. The double mutant, pET31f1mMCA5Y64A/Y131A, was produced by ligating the 301 bp Pst I fragment from pET31f1mMCA5cY64A into a backbone derived from pET31f1mMCA5cY131A with the 301 bp Pst I fragment removed. All of the truncated and mutated sequences were confirmed by DNA sequencing.

Expression and Purification of Mouse CA V

The levels of CA V production from the CA V expression vectors ranged from 1 to 4 mg / liter of culture. Lengthening the time of incubation after induction to six hours increased yields by 20-50%. Electrophoresis of the purified enzymes on an SDS-polyacrylamide gels showed that mouse CA Vb and Vc were >95% pure and had the expected molecular masses of 31 and 28 kDa (Figure 3-2). The expressed enzymes CA Va, Vb, and Vc were amino terminally sequenced. Analysis of several preparations of CA Va indicated

that it was a mixed fraction with 18-51 residues of the expected NH₂-terminal sequence removed by *E. coli* proteases, even in the presence of several protease inhibitors during the lysis and purification. CA Vb had the expected amino terminal residues; CA Vc had the initial methionine removed (Figure 3-1).

Discussion

The nucleotide sequence reported by Amor-Gueret and Levi-Strauss was found to differ from the nucleotide sequence we isolated from cDNA for mouse CA V. Five of the differences, three silent mutations and two resulting in amino acid changes as well, appear to be simple point mutations that may be the result of strain differences between the mice used (Figure 3-3). The nucleotide sequence of the mouse CA V cDNA reported by Ngao *et al.* (1994) matches the sequence of Amor-Gueret and Levi-Strauss (1990) at these five positions. The two amino acid sequence alterations at nucleotide positions 643 and 764 of Figure 3-3 result in somewhat conservative amino acid substitutions of leucine to methionine and threonine to methionine. The crystal structure of CA Vc (Boriack *et al.*, 1995) places these residue at the surface of the enzyme, their side-chains extending into

solution and not participating in any obvious secondary or tertiary structural interactions.

However, the sequence differences noted at positions 451-459 (Figure 3-3) do occur within a structurally and functionally important region of CA V. The insertion of three nucleotides in this region restores an amino acid sequence, valine-histidine-tryptophan, that is highly conserved in nearly all of the mammalian carbonic anhydrase isozymes (see Figure 1-1, amino acid positions 121-123). Ngao *et al.* (1994) also reported a nucleotide sequence which restored the valine-histidine-tryptophan motif for CA V cDNA isolated from C57BL mice. The crystal structure of CA Vc (Boriack *et al.*, 1995) places these residues along the hydrophobic cavity within the active site. While it is possible that the sequence reported by Amor-Gueret and Levi-Strauss (1990) in this region is correct and represents a strain difference between B10.HTT mice and BALB/c or C57BL, it is more likely due to an sequencing error or cloning artifact since the sequence motif, Val-His-Trp, is found in nearly all of the carbonic anhydrase isozymes and is located within the active site.

I expressed and purified three types of mouse carbonic anhydrase V (forms Va, Vb, and Vc; Figure 3-1) that represent possible isoforms of this enzyme in the cytoplasm and mitochondria. CA Va is a potentially full-length protein product based on the complete cDNA sequence (Amor-Gueret and

Levi-Strauss, 1990). Electrophoretic analysis and amino-terminal sequencing (Figures 3-1 and 3-2) show that this protein is a mixture of at least four species, with loss of between 18 and 51 amino acids. The initial 29 amino acid residues of mouse CA Va shown in Figure 3-1 are presumably part of a mitochondrial targeting sequence and should play no role in catalysis (as will be demonstrated in Chapter 4 of this work). The observation that many cleavages can occur within the first 50 amino acids of the NH₂-terminus of CA V when it is expressed in *E. coli* may be an indication that this region is very susceptible to proteolysis. SDS-PAGE analysis of several of the MCA Va mutants also revealed a mixture of smaller than expected proteins.

The CA Vb and CA Vc proteins, and all the mutants derived from these proteins, have been produced as pure, homogeneous preparations in milligram quantity for many types of analysis. Amino terminal analysis of CA Vb and CA Vc indicates that these proteins have the expected amino termini except for the removal of the initial methionine in MCA Vc.

MCA Vc that I prepared has been crystallized and its xray crystal structure has been solved at a resolution of 2.45 angstroms (Boriack *et al.*, 1995) and shown to be highly similar to the structure for human CA II.

CHAPTER 4 KINETIC AND INHIBITION PROPERTIES OF CARBONIC ANHYDRASE V

Introduction

Although a mitochondrial carbonic anhydrase activity was first described over 35 years ago (Datta and Shepard, 1959), little is known about the kinetic or inhibition properties of the isozyme now known as carbonic anhydrase V. Comparison of the primary sequence of CA V with that of CA I, II, and III at positions which have been shown to directly affect activity suggests that CA V may have significantly different properties than the other isozymes of carbonic anhydrase.

The turnover number (k_{cat}) for CA V from rat kidney mitochondria has been reported as $24,000\text{ s}^{-1}$ when measured at 37°C and a pH of 7.4 (Dodgson and Cherian, 1989). This turnover number is larger than that of human CA I, with a maximal value of k_{cat} of $10,000\text{ s}^{-1}$ and substantially less than the values determined for human CA I and CA II of $2 \times 10^5\text{ s}^{-1}$ and $1.4 \times 10^6\text{ s}^{-1}$ determined at 25°C (Khalifah, 1971; Jewell *et al.*, 1991). However, the activity of CA V has been reported to be highly sensitive to pH, with an 8-fold

increase in CA V activity from pH 7 to 8 (Dodgson *et al.*, 1982). Therefore the maximal turnover number of CA V is presumably higher than $24,000 \text{ s}^{-1}$.

Numerous studies (reviewed by Dodgson (1991)) have suggested that mitochondrial anhydrase activity is used in the metabolic pathways of gluconeogenesis and ureagenesis to provide a ready source of bicarbonate as substrate for carbamoyl phosphate synthetase and pyruvate carboxylase. Most of this data has been based upon the correlation of the effect of sulfonamide inhibitors such as ethoxzolamide or acetazolamide upon CA V activity and upon the synthesis of citrulline, urea, and glucose. These studies have been difficult to interpret quantitatively in many cases due to the possibility of other carbonic anhydrase isozymes being present and a paucity of CA V inhibition constants.

Stopped-flow spectrophotometry and ^{18}O exchange were used to measure the catalytic rate constants for full length and truncated forms of mouse carbonic anhydrase V synthesized in a bacterial expression system. I have determined the maximal steady state constants for the hydration of CO_2 to be substantially higher than previously reported for CA V, with maximal values very similar to carbonic anhydrase I. The pH-rate profile for k_{cat} indicates that CA V is quite sensitive to pH, not achieving a maximal turnover number until pH is higher than 9. A comparison the kinetic properties of MCA

Vb and MCA Vc was made to show that the different amino termini of these isoforms do not affect catalytic activity. The inhibition constants of ethoxzolamide, acetazolamide, and cyanate were determined to be similar to CA II, with K_i 's for ethoxzolamide and acetazolamide in the low nanomolar range.

Material and Methods

Measurement of Catalysis at Steady State

Initial velocities of CO_2 hydration were measured by stopped-flow spectrophotometry (Applied Photophysics Model SF.17MV) following the change in absorbance of a pH indicator at 25° C. (Khalifah, 1971). Solutions of CO_2 were prepared by bubbling CO_2 into water under controlled conditions for which the concentration of saturated CO_2 is known. Dilutions were done between syringes with a gas-tight connection. Final concentrations of CO_2 were varied from 0.5 to 17 mM. The buffer-indicator pairs, their pK_a values, and the wavelength observed were as follows; Mes ($\text{pK}_a = 6.1$) with chlorophenol red ($\text{pK}_a = 6.3$), 574 nm; Mops ($\text{pK}_a = 7.2$) with *p*-nitrophenol ($\text{pK}_a = 7.1$), 400 nm; Hepes ($\text{pK}_a = 7.5$) with phenol red ($\text{pK}_a = 7.5$), 557 nm; Taps ($\text{pK}_a = 8.4$) with *m*-cresol purple ($\text{pK}_a = 8.3$), 578 nm; Ches ($\text{pK}_a = 9.3$) with thymol blue ($\text{pK}_a = 8.9$), 590 nm. Solutions were maintained at a constant

total ionic strength of 0.2 M by addition of the appropriate amount of sodium sulfate. The mean initial rate in each case was determined from at least six reaction traces comprising the initial 5-10% of the reaction. Uncatalyzed rates were subtracted, and determination of the kinetic constants k_{cat} and k_{cat}/K_M was by nonlinear least-squares methods (Enzfitter, Elsevier-Biosoft).

Oxygen-18 Exchange Kinetics

The rate of exchange of ^{18}O between CO_2 and water and the rate of exchange of ^{18}O from $^{12}\text{CO}_2$ to $^{13}\text{CO}_2$ caused by the transitory labeling of the active site of carbonic anhydrase were determined using the method described by Silverman (1982). Both of these exchanges were observed at chemical equilibrium. The reaction solution was in contact with a membrane permeable to gases; CO_2 passing across the membrane entered a mass spectrometer (Extrel EXM-200) providing a continuous measure of isotopic content of CO_2 . Experiments were performed without added buffers. Solutions were maintained at a constant total ionic strength of 0.2 M by the addition of the appropriate amount of sodium sulfate.

The ^{18}O exchange method is useful because it measures the rate of interconversion of CO_2 and HCO_3^- at chemical equilibrium, R_1 , as shown in Equation 5.



The substrate dependence of R_i is given by $R_i/[E] = k_{\text{cat}}^{\text{ex}}[S]/(K_{\text{eff}}^{\text{S}} + [S])$, where $k_{\text{cat}}^{\text{ex}}$ is a rate constant for maximal interconversion of CO_2 and HCO_3^- , $K_{\text{eff}}^{\text{S}}$ is an apparent substrate binding constant, and $[S]$ is the concentration of CO_2 or HCO_3^- or both (Simonsson *et al.*, 1979). Values of $k_{\text{cat}}^{\text{ex}}/K_{\text{eff}}^{\text{S}}$ for the enzymes were determined by nonlinear least-squares fit of the above expression for R_i to the data for varying substrate concentration or by measurement of R_i at values of $[S]$ much smaller than $K_{\text{eff}}^{\text{S}}$. In theory and in practice, $k_{\text{cat}}^{\text{ex}}/K_{\text{eff}}^{\text{CO}_2}$ is equal to $k_{\text{cat}}/K_{\text{M}}$ for the hydration of CO_2 obtained by steady-state methods (Simonsson *et al.*, 1979; Silverman, 1982).

Values of K_i were obtained from ^{18}O exchange experiments and were determined from the least-squares fit of R_i at various inhibitor concentrations to the expression for mutual depletion of free enzyme and inhibitor (Segel, 1975). Since the total substrate concentration ($[\text{CO}_2] + [\text{HCO}_3^-] = 25 \text{ mM}$) is much less than $K_{\text{eff}}^{\text{S}}$ ($>100 \text{ mM}$) for these experiments, we cannot differentiate between competitive and noncompetitive modes of inhibition.

Results

Measurement of Catalytic Activity

The catalytic constant k_{cat}/K_M for CO_2 hydration catalyzed by CA Vc (the shortest form; see Figure 3-1) was determined by stopped-flow spectrophotometry and by ^{18}O exchange measured by mass spectrometry. Over the range of pH 5-9, k_{cat}/K_M determined from ^{18}O exchange could be described as dependent on the unprotonated state of a single ionizable group with $\text{pK}_a = 7.4 \pm 0.1$ and a maximum of $(3.5 \pm 0.1) \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (Figure 4-1). The results for k_{cat}/K_M obtained by stopped-flow spectrophotometry were $\text{pK}_a = 7.8 \pm 0.1$ and a maximum of $(3.2 \pm 0.3) \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. The values of k_{cat}/K_M and apparent pK_a obtained by stopped-flow spectrophotometry and ^{18}O exchange for the longer form, CA Vb, were identical to those obtained for CA Vc. The longest form, CA Va, was subject to proteolysis, and I was not able to isolate a pure form for kinetic analysis. While the concentration of active sites could be determined by titration with an inhibitor, purified samples of CA Va were considered to be heterogeneous mixtures of CA V with amino terminal truncations. However, the catalytic activity of these mixtures was not appreciably different from that of the two purified shorter forms, suggesting

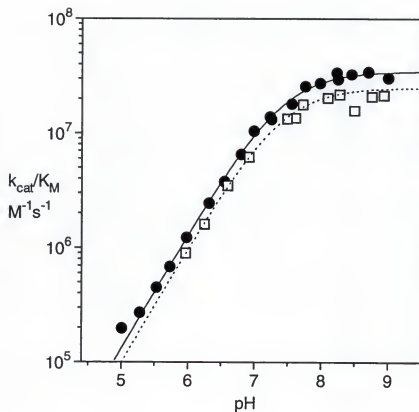


Figure 4-1. k_{cat}/K_M catalytic constants for hydration of CO_2 catalyzed by mouse carbonic anhydrase Vc ($\bullet$) and Vb ($\square$). These data were obtained at 25°C using ^{18}O exchange measured in the absence of buffers. The dotted line (for CA Vb) is a least-squares fit to a single ionization with $\text{pK}_a = 7.4 \pm 0.1$ and a maximal value of $k_{cat}/K_M = (2.5 \pm 0.1) \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. The solid line (for MCA Vc) is a fit with $\text{pK}_a = 7.4 \pm 0.1$ and a maximal value of $k_{cat}/K_M = (3.5 \pm 0.1) \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$.

that the presence of portions of the amino terminal region in the longer form do not affect catalysis.

The values of k_{cat} for the hydration of CO_2 for the two forms CA Vb and Vc showed a monotonic increase with pH. In each case, measurement of a maximal value of k_{cat} was hampered by the loss of activity for enzymes placed in solutions with a pH above 9.5. For CA Vb, there was only a hint of reaching a maximum, with values of k_{cat} near $3 \times 10^5 \text{ s}^{-1}$ at pH 9.5. Extended work with CA Vc gave the clearest indication of the maximal value of k_{cat} at $(3.2 \pm 0.3) \times 10^5 \text{ s}^{-1}$ and an apparent pK_a of 9.2 ± 0.2 (Figure 4-2). The steady-state constants for CA V and comparisons with isozymes I, II, and III are given in Table 4-1. Comparison of the inhibition of these enzymes by the sulfonamides acetazolamide and ethoxzolamide, as well as by cyanate, are given in Table 4-2.

Discussion

I have found that the two shorter forms of CA V (forms Vb and Vc; Figure 4-1) have nearly the same catalytic properties in the hydration of CO_2 . This is consistent with the report that a deletion mutant of HCA II lacking 24 residues at the NH_2 -terminus is nearly fully active (Aronsson *et al.*, 1995). This segment of CA II (as well as of CA I and III) contains Tyrosine 7, the side chain of which extends into the active-site cavity and is a component of the

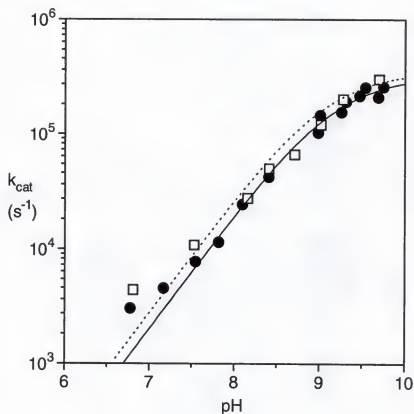


Figure 4-2. k_{cat} catalytic constants for hydration of CO_2 catalyzed by mouse carbonic anhydrase Vc (●) and Vb (□). These data were obtained at 25° C using stopped-flow measurements. The dotted line (for CA Vb) is a least-squares fit to a single ionization with $\text{pK}_a = 9.1 \pm 0.3$ and a maximal value of $k_{cat} = (3.5 \pm 0.4) \times 10^5 \text{ s}^{-1}$. The solid line (for MCA Vc) is a fit with $\text{pK}_a = 9.2 \pm 0.2$ and a maximal value of $k_{cat} = (3.2 \pm 0.3) \times 10^5 \text{ s}^{-1}$.

Table 4-1

Maximal Values Of The Steady-State Constants For The Hydration Of CO_2 Catalyzed By Isozymes Of Carbonic Anhydrase At 25° C

CA	k_{cat} (s^{-1})	k_{cat}/K_M ($\text{M}^{-1} \text{s}^{-1}$)	Ref.
Human I	2×10^5	5×10^7	Khalifah (1971)
Human II	1.4×10^6	1.5×10^8	Khalifah (1971)
Human III	1×10^4	3×10^5	Jewel <i>et al.</i> (1991)
Mouse V ^a	3×10^5	3×10^7	This work

^a These values are for the two short forms of CA V designated b and c in Figure 3-1.

Table 4-2

Inhibition of Isozymes of Carbonic Anhydrase Measured by ^{18}O Exchange at pH 7.2-7.5 and 25° C

CA	K_i		
	Acetazolamide	Ethoxzolamide	Cyanate
	mM		
Human I ^a	0.2	0.002	0.7
Human II	0.06	0.008	30
Human III	40	8	30
Mouse Vc ^b	0.06	0.005	30

The inhibition data were obtained using a least-squares fit to the expression for mutual depletion of inhibitor and free enzyme (Segel, 1975) with total substrate concentration much less than the apparent binding constant of substrate to enzyme ($[\text{S}] \ll K_{\text{eff}}^{\text{S}}$)

^a These data for HCA I are taken from Sanyal *et al.* (1982) and were measured at 0° C for acetazolamide and ethoxzolamide and at 25° C for cyanate.

^b These values are for CA Vc, the shortest form of CA V (Figure 3-1).

hydrogen-bonded array of side chains and water molecules found in the active-site cavity of the crystal structure (Eriksson *et al.*, 1988). Mouse and human CA V contain a histidine or threonine at position 7, respectively. It seems clear that the absence of residue 7 and the NH_2 -terminal segment in which it is found do not contribute significantly to the catalytic pathway under the conditions of this study. This is further emphasized by the result from kinetic analysis that mixtures of the longer forms of CA V, obtained from proteolytic products of CA Va such as shown in Figure 3-2, had catalytic activity equivalent to that of CA Vb and Vc.

The maximal values of the kinetic constant k_{cat}/K_m for the hydration of CO_2 for CA Vb and Vc were $3 \times 10^7 \text{ M}^{-1} \text{ S}^{-1}$. The pH profile of k_{cat}/K_m appeared to be dependent on the basic form of a single ionizable group with apparent pK_a near 7.4 (Figure 4-1). This pK_a has been shown to represent the ionization of the zinc-bound water (Simonsson and Lindskog, 1982), which in isozyme II is close to 7, although it is dependent on experimental conditions and the ionization state of the nearby Histidine 64 (Simonsson and Lindskog, 1982). Thus, isozyme V has an apparent pK_a for this zinc-bound water close to that of isozyme II. A comparison of maximal values of k_{cat}/K_m for the hydration of CO_2 catalyzed by several isozymes is shown in Table 4-1.

A significant difference in catalysis by isozymes I and II compared with isozyme V was found in k_{cat} for hydration. For CA I and II, k_{cat} is described by a titration curve with a pK_a near 7 reaching a maximum at high pH (Khalifah, 1971; Steiner *et al.*, 1975). In CA II, this behavior is a reflection of the pK_a of histidine 64, the proton acceptor in the intramolecular proton shuttle that transfers a proton to solution and regenerates the zinc-bound hydroxide (Steiner *et al.*, 1975; Silverman and Lindskog, 1988). In mouse CA Vb and Vc, we observed an increase in k_{cat} for hydration with an increase in pH and an apparent pK_a of 9.2 (Figure 4-2). However, the maximal values of k_{cat} for the hydration of CO_2 by mouse CA Vb and Vc are near $3 \times 10^5 \text{ s}^{-1}$, a very sizable value. Together, these data suggest the presence of a proton acceptor in the active-site cavity with a pK_a near 9. There could be more than one proton acceptor, of course, but there does not appear to be a predominant proton acceptor in the initial 51 residues of CA Va (Figure 3-1) since the deletion of the segment leaves k_{cat} relatively unchanged.

One prominent difference in the active-site cavities of CA V and II is at position 64. The histidine at this position in human CA II has been shown to act as a proton shuttle, accepting protons from the zinc-bound water and transferring them to solution (Tu *et al.*, 1989). The presence of Histidine 64 in CA II is responsible for the apparent pK_a near 7 in k_{cat} for the hydration of CO_2

(Tu *et al.*, 1989; Steiner *et al.*, 1975). CA V has a tyrosine at position 64 that is a poor proton shuttle at pH 7, but is a more efficient shuttle at pH near the pK_a of the phenolic hydroxyl.

CHAPTER 5
PROTON TRANSFER AND THE EFFECTS OF ACTIVE SITE RESIDUES
64,65, AND 131 UPON THE CATALYTIC ACTIVITY OF CARBONIC
ANHYDRASE V

Introduction

The catalytic properties of carbonic anhydrase V appear similar to those of the well studied isozymes CA I and CA II, except for an unusually high pH sensitivity. CA V's maximal rate constants for the hydration of CO_2 are similar to those for CA I and about 20% as high as CA II (Table 4-1), one of the fastest enzymes known. The apparent pK_a near 7 for the zinc-bound water, as determined by pH-rate profiles of k_{cat}/K_M , is quite similar for CA I, II, and V.

Mitochondrial carbonic anhydrase activity has been reported as being very sensitive to pH, with an 8-fold increase in CA activity from pH 7 to 8 compared with a 2-fold increase for erythrocyte CA (Dodgson *et al.*, 1980; Dodgson *et al.*, 1982). Careful measurements of the pH rate profiles for turnover number, k_{cat} , of CA V as shown in Figure 4-2 support these earlier observations. The measured values of k_{cat} increase 5-fold from pH 7.2 to pH 8.1 and 9-fold from pH 7 to pH 8 when plotted on the best-fit line for the data.

The increase in k_{cat} with increasing pH appears dependent upon the ionization of a residue in the active site of CA V with a pK_a greater than 9.

In carbonic anhydrase II the rate limiting step for the hydration of CO_2 is the transfer of a proton from the zinc-bound water to solution to generate the zinc-bound hydroxide. Steiner *et al.* (1975) and Pocker and Bjorkquist (1977) demonstrated that CA II has a solvent deuterium isotope effect between 3-4 for V_{max} and an isotope effect of 1 for V_{max}/K_M . Steiner *et al.* (1975) proposed that this was an indication that the proton transfer step was rate limiting and therefore V_{max} was affected by deuterium, but not V_{max}/K_M . The assumption that proton transfer is rate limiting in the catalytic mechanism for CA V was confirmed by measuring the solvent hydrogen isotope effects upon CA V catalytic activity.

The value of k_{cat} in CA II is dependent upon the ionization of a residue with an apparent pK_a near 7 (Pocker and Bjorkquist, 1977). This residue was shown to be histidine 64 by site specific mutagenesis (Tu *et al.*, 1989). Carbonic anhydrase V has a tyrosine at position 64. The pK_a for the ionization of the phenolic hydroxide of tyrosine is consistent with the much higher apparent pK_a observed for pH profiles of k_{cat} in CA V. To test if the tyrosine at position 64 of CA V is necessary for proton transfer, the catalytic activity of a

mutant with an alanine at position 64 was compared with that of the wild type enzyme.

Jewel *et al.* (1991) have shown that a proton transfer site can be introduced into CA III by substituting a histidine for the naturally occurring lysine at position 64. A Y64H mutant of CA V was tested to see if the catalytic properties of CA V could be altered to more closely resemble those of CA II.

A detailed examination of the crystal structure of CA V (Boriack *et al.*, 1995) reveals only five ionizable residues within 12 Å of the zinc bound water which may act as proton acceptors: histidine 7, tyrosine 64, lysine 91, tyrosine 131, and lysine 132. MCA Vc, the deletion mutant lacking residues 1-21, has previously been shown to function similarly to MCA Vb, implying that histidine 7 is not necessary for proton transfer. Tyrosine 131 fulfills several of the criteria for a potential proton transfer site listed by Boriack *et al.* (1995); 1) the pK_a of tyrosine is close to the pH of the biological environment of the enzyme, 2) the hydroxide of tyrosine 131 is less than 10 Å from the zinc-bound water, and 3) tyrosine 131 has a solvent accessible surface area greater than 85 Å². Mutants of CA V with an alanine or a histidine at position 131 were created and their pH profiles measured to test if changes at this position affected the catalytic rate of CA V. In order to test the possibility that both tyrosine 64 and

tyrosine 131 could facilitate proton transfer, a double mutant with alanines at positions 64 and 131 was also constructed and tested.

The phenylalanine at position 65 of carbonic anhydrase V is unusual among the isozymes of carbonic anhydrase. Boriack *et al.* (1995) have shown that this residue sterically hinders the access of position 64 to the active site. The Y64H CA V mutant had catalytic properties very similar to the wild type CA V. To test if proton transfer from a histidine at position 64 could be enhanced by reducing the size of the adjacent residue at position 65, the catalytic activity of Y64H/F65A CA Vc was compared to that of CA V and Y64H CA V.

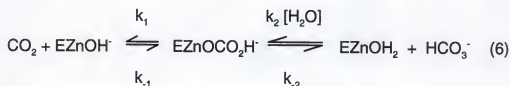
Materials And Methods

Wild type and mutant forms of mouse carbonic anhydrase V were cloned, mutated, and expressed as described in Chapter 3. Stopped flow spectrophotometry and ^{18}O exchange were used to determine the steady state and equilibrium rates for the hydration of CO_2 and the dehydration of HCO_3^- as described in Chapter 4.

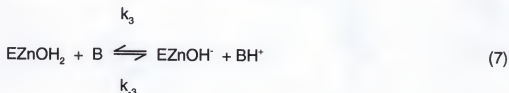
Results

Interconversion of Carbon Dioxide and Bicarbonate

For the hydration of CO_2 catalyzed by carbonic anhydrases, the ratio of k_{cat}/K_M contains rate constants from the binding of CO_2 to the departure of HCO_3^- and describes the steps in the conversion of CO_2 and HCO_3^- (eq. 6).



The zinc-bound hydroxide is regenerated in a separate step (7) in which a proton is transferred from the zinc-bound water to a proton acceptor, B, which can be water in the active-site cavity, a side chain of the enzyme such as histidine 64 in CA II, or a small buffer molecule (Kararli and Silverman, 1985; Tu *et al.*, 1990; Paranawithana *et al.*, 1990).



The values of k_{cat}/K_M reported in Figures 5-1 and 5-2 were determined by ^{18}O exchange in the absence of buffers. The maximal value of k_{cat}/K_M and the apparent pK_a for catalysis are listed in Table 5-1. These values were determined by a least squares fit of the data to equation 8.

$$k_{\text{cat}}/K_M = K_E M / ([\text{H}^+] + K_E) \quad (8)$$

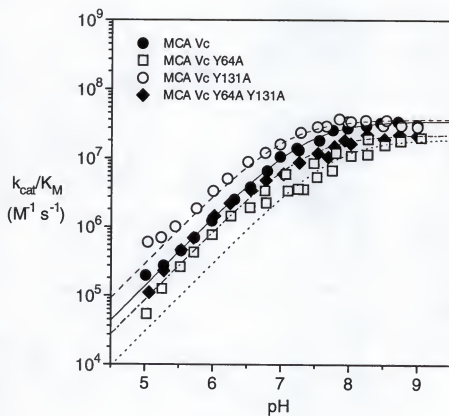


Figure 5-1. ^{18}O exchange k_{cat}/K_M catalytic constants for hydration of CO_2 catalyzed by mouse carbonic anhydrase Vc (●) mutants Y64A CA Vc (□), Y131A CA Vc (○), and Y64A/Y131A CA Va (◆). The lines are least-squares fits of the data with values of apparent pK_a and maximal k_{cat}/K_M given in Table 5-1.

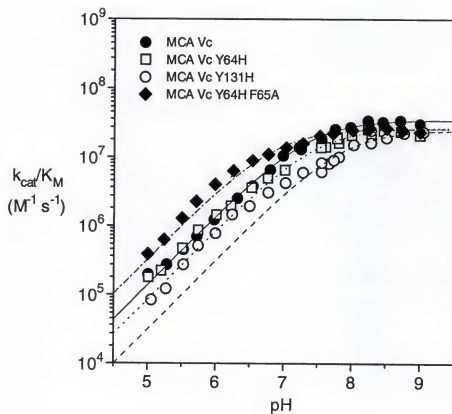


Figure 5-2. ^{18}O exchange k_{cat}/K_M catalytic constants for hydration of CO_2 catalyzed by mouse carbonic anhydrase Vc (●) mutants Y64H CA Vc (□), Y131H CA Vc (○), and Y64H/F65A CA Va (◆). The lines are least-squares fits of the data with values of apparent pK_a and maximal k_{cat}/K_M given in Table 5-1.

Table 5-1

Apparent pK_a and Maximal Values of k_{cat}/K_M Stopped Flow and ^{18}O Exchange measurements of Catalysis by Mouse Carbonic Anhydrase V and Mutants of CA V.

enzyme	^{18}O Exchange		stopped flow	
	k_{cat}/K_M ($\times 10^7 M^{-1} s^{-1}$)	pK_a	k_{cat}/K_M ($\times 10^7 M^{-1} s^{-1}$)	pK_a
CA Vc	3.5 ± 0.1	7.4 ± 0.1	2.5 ± 0.1	7.4 ± 0.1
Y64A	1.9 ± 0.1	7.8 ± 0.1	3.1 ± 0.3	8.8 ± 0.2
Y131A	3.7 ± 0.1	7.1 ± 0.1	6.4 ± 0.4	8.2 ± 0.1
Y64A/Y131A	2.2 ± 0.1	7.4 ± 0.1	6.1 ± 0.2	8.3 ± 0.1
Y64H	2.7 ± 0.1	7.5 ± 0.1	4.6 ± 0.1	8.5 ± 0.1
Y131H	2.5 ± 0.1	7.9 ± 0.1	1.0 ± 0.1	8.6 ± 0.1
Y64H/F65A (c)	2.6 ± 0.1	6.9 ± 0.1	6.6 ± 0.4	8.1 ± 0.1

Apparent pK_a and maximal k_{cat}/K_M are calculated from the least squares fit to a single ionization.

K_E is the apparent ionization constant and M is the maximal value of k_{cat}/K_M .

When determined by ^{18}O exchange at equilibrium, the k_{cat}/K_M pH profiles for all of the mutants tested were qualitatively similar to that of the wild type enzyme (Figures 5-1, 5-2). Table 5-1 shows the largest deviation of maximal k_{cat}/K_M from wild type was a 46% reduction for Y64A CA Vc and the apparent pK_a determined from these pH profiles agreed to within 0.5 pH units of the apparent pK_a for the wild type CA V enzyme. The highest pK_a determinations were from the pH profiles of Y64A CA Vc and Y131H, with values for pK_a of 7.8 and 7.9 respectively.

Stopped-flow measurements of CO_2 hydration at steady state were also used to determine the pH profiles of k_{cat}/K_M shown in Figures 5-3 and 5-4 and the values of maximal k_{cat}/K_M and apparent pK_a listed in Table 5-1. These pH profile determinations of k_{cat}/K_M were less consistent and more complex than those from ^{18}O exchange measurements made at equilibrium. All of the mutant enzymes had an apparent pK_a that was significantly higher (from 8.1 to 8.8, Table 5-1) than the observed pK_a for wild type CA Vc ($pK_a = 7.4$) and the apparent pK_a measured by ^{18}O exchange for each mutant (from 6.9 to 7.9). The differences in apparent pK_a determined by ^{18}O exchange and stopped flow

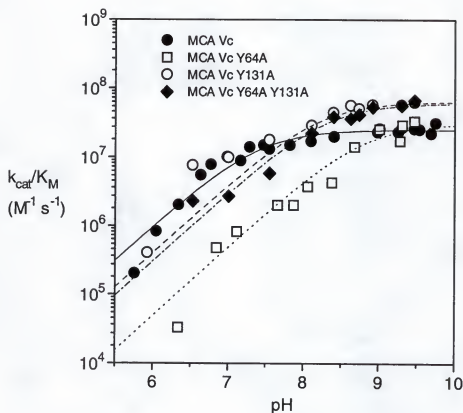


Figure 5-3. Stopped-flow k_{cat}/K_M catalytic constants for hydration of CO_2 catalyzed by mouse carbonic anhydrase Vc (●) mutants Y64A CA Vc (□), Y131A CA Vc (○), and Y64A/Y131A CA Va (◆). The lines are least-squares fits of the data with values of apparent pK_a and maximal k_{cat}/K_M given in Table 5-1.

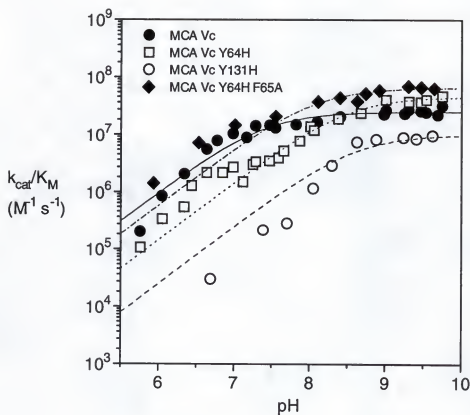


Figure 5-4. Stopped-flow k_{cat}/K_M catalytic constants for hydration of CO_2 catalyzed by mouse carbonic anhydrase Vc (●) mutants Y64H CA Vc (□), Y131H CA Vc (○), and Y64H/F65A CA Va (◆). The lines are least-squares fits of the data with values of apparent pK_a and maximal k_{cat}/K_M given in Table 5-1.

(from 0.7 to 1.2) were consistent; the mutants with the highest apparent pK_a for k_{cat}/K_M from stopped flow also had the highest pK_a from ^{18}O exchange.

For the mutants Y131A CA Vc, Y64H CA Vc, and Y64H F65A cA Vc, the pH profiles did not fit a line describing a single pK_a well at lower pH and may describe a dependence of k_{cat}/K_M upon more than a single ionization event.

Proton Transfer

To investigate the importance of proton transfer upon the catalytic properties of CA V, site specific mutants of potential proton transfer sites were tested for alterations in turnover number. Stopped flow spectrophotometry was used to determine the turnover number at steady state. The mutants were designed to test the effects of alanine and histidine residues substituted for the tyrosines at positions 64 and 131 in carbonic anhydrase V.

Figure 5-5 compares the pH profiles of k_{cat} for mutants with alanines substituted for the tyrosines at positions 64 or 131 or both. The substitution of an alanine for either tyrosine 64 or 131 did not affect the apparent pK_a of 9.2 observed in the wild type CA Vc. The apparent pK_a observed for the double mutant, Y64A/Y131A CA Vc, was slightly decreased to 8.8, but there was still a strong pH dependence for this double mutant with alanines at positions 64 and 131. The maximal turnover numbers determined for the mutants were

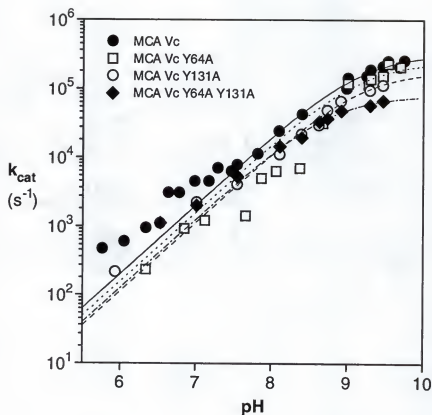


Figure 5-5. Stopped-flow k_{cat} catalytic constants for hydration of CO_2 catalyzed by mouse carbonic anhydrase Vc (●) mutants Y64A CA Vc (□), Y131A CA Vc (○), and Y64A/Y131A CA Va (◆). The lines are least-squares fits of the data with values of apparent pK_a and maximal k_{cat} given in Table 5-2.

decreased from that of the wild type enzyme (Table 5-2). The total decrease from CA Vc in maximal k_{cat} for Y64A/Y131A CA Vc ($2.4 \times 10^5 \text{ s}^{-1}$) is near to the sum of the decreases for Y64A CA Vc and Y131A CA Vc ($0.7 \times 10^5 \text{ s}^{-1} + 1.4 \times 10^5 \text{ s}^{-1}$).

A second approach to determine if a specific site of CA V could participate in proton transfer was to place a histidine at position 64 or 131. The pH profiles for k_{cat} for these mutants are shown in Figure 5-6. The substitution of a histidine for either tyrosine 64 or 131 did not affect the apparent pK_a of 9.2 observed in the wild type CA Vc. The maximal turnover number for Y131H was significantly reduced, with the lowest value ($6 \times 10^4 \text{ s}^{-1}$) seen for any of the mutants of CA V used in this study as shown in Table 5-2. The value of k_{cat} for Y64H CA Vc was slightly elevated over wild type, particularly below pH 7.5. A line describing two ionizations with pK_a of 9.3 and 6.2 and maxima of $3.8 \times 10^5 \text{ s}^{-1}$ and $6 \times 10^3 \text{ s}^{-1}$ is shown with the data for Y64H CA Vc.

To test the influence of phenylalanine 65 upon position 64, a double mutant, Y64H/F65A was constructed. This double mutant had a substantial increase in catalytic activity as shown in Figure 5-6. The maximal k_{cat} was twice that of the wild type CA V. The increase in k_{cat} was even more pronounced at lower pH with an approximately 80-fold increase over wild type

Table 5-2
Apparent pK_a and Maximal Values of k_{cat} and R_{H₂O} for Mouse Carbonic Anhydrase V and Mutants of CA V.

enzyme	k _{cat} (x10 ⁵ s ⁻¹)	pK _a
CA Vc	3.2 ± 0.2	9.2 ± 0.1
Y64A	2.5 ± 0.5	9.2 ± 0.1
Y131A	1.8 ± 0.2	9.2 ± 0.1
Y64A/Y131A	0.8 ± 0.1	8.8 ± 0.1
Y64H	3.8 ± 0.6	9.2 ± 0.1
Y131H	0.6 ± 0.1	9.2 ± 0.1
Y64H/F65A	7.5 ± 0.7 ^a (1.5 + 5.9) ^b	6.3, 9.0 ^a

k_{cat} values were obtained from stopped flow measurements at steady state. All measurements were made at 25° C. Apparent pK_a is calculated from the best fit line for a single ionization for k_{cat} determined by nonlinear regression analysis.

^a The data was fit to a line describing two ionizations

^b Two maxima were observed and summed to arrive at 7.5 x 10⁵.

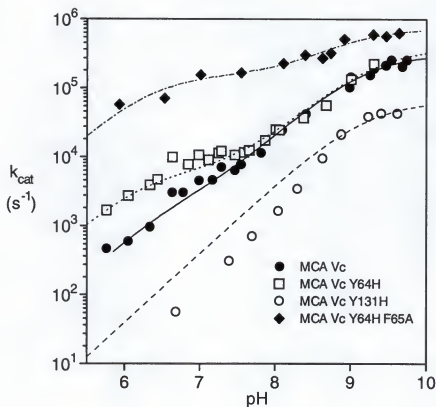


Figure 5-6. Stopped-flow k_{cat} catalytic constants for hydration of CO_2 catalyzed by mouse carbonic anhydrase Vc (●) mutants Y64H CA Vc (□), Y131H CA Vc (○), and Y64H/F65A CA Va (◆). The lines are least-squares fits of the data with values of apparent pK_a and maximal k_{cat}/K_M given in Table 5-2.

seen at pH 7. The data for Y64H/F65A CA Vc could be fit by nonlinear regression to a line describing the ionization of two moieties with pK_a of 6.3 and 9.0 and maxima of $1.5 \times 10^5 \text{ s}^{-1}$ and $5.9 \times 10^5 \text{ s}^{-1}$.

Discussion

Measurements of the catalytic rate constants for carbonic anhydrase V, as well as data from sequence and structure comparisons with other isozymes of carbonic anhydrase, suggest that the elevated pH sensitivity of CA V may arise from differences in its proton transfer pathway compared to other isozymes. The importance of tyrosine 64, phenylalanine 65, and tyrosine 131 in proton transfer were by determined by testing the effects of histidine or alanine substitutions at these positions.

A histidine at position 64 of wild type CA II or a mutant of CA III is necessary for intramolecular proton transfer to occur (Tu *et al.*, 1989; Jewel *et al.*, 1991). Replacement of histidine 64 with alanine in CA II results in a greater than 10-fold decrease in the proton transfer dependent rate, k_{cat} , and eliminates the pH dependence of this rate compared to the wild type enzyme (Tu *et al.*, 1989). Replacement of tyrosine 64 with alanine in CA V did not eliminate the pH dependence of k_{cat} seen in the wild type CA V and only caused a modest decrease in their maximal values. Analysis of the crystal

structure of CA V has suggested that tyrosine 131 in CA V may be able to function similarly to histidine 64 in CA II as a proton transfer site at pH values near its pK_a (Boriack *et al.*, 1995). However, the Y131A CA Vc mutant had a pH profile for k_{cat} very much like that of the wild type CA V. A double mutant, Y64A/Y131A CA Vc, also retained the characteristically high pH dependence seen for wild type CA V. The lack of effect of alanine substitutions at tyrosine 64 or tyrosine 131 could be an indication that neither residue is necessary for proton transfer to occur.

It may be possible that proton transfer can be facilitated by multiple pathways in CA V and that the elimination of one pathway is masked by the presence of others. The decreases in maximal values of k_{cat} for these alanine substitutions weakly supports this hypothesis, but implies that there are additional residues that can function as proton transfer sites. The most likely possibilities are lysine 131, lysine 132, and lysine 91. These lysine residues, like tyrosine, may have a pK_a near the apparent pK_a observed for the pH profile of k_{cat} seen in the Y64A/Y131A CA Vc mutant.

A second approach to determining if positions 64 or 131 in CA V could function in a proton shuttle was to replace the tyrosines at either position with a histidine and see if the pH dependence was shifted toward the pK_a of histidine. The placement of histidine at position 64 in CA V did not

significantly alter the pH profiles as compared to the wild type enzyme, with the exception of a small increase in k_{cat} at pH values below 7.5. The lack of proton transfer activity involving a tyrosine or a histidine at position 64 may be partly explained by the presence of a bulky phenylalanine at position 65 which sterically prohibits residues at position 64 from interacting with water in the active site of CA V (Boriack *et al.*, 1995). The double mutant, Y64H/F65A CA Vc, demonstrated that histidine 64 with an adjacent alanine 65 has substantially increased values for turnover number, particularly at a pH nearer to the pK_a expected for a histidine. These results indicate that a histidine at position 64 of CA V is prevented from being a major component of a proton shuttle by the presence of phenylalanine 65. A F65A CA Vc mutant needs to be tested to see if tyrosine 64 can also increase the rate of catalysis in the absence of phenylalanine 65.

The replacement of tyrosine 131 with histidine in CA V may have altered more than just proton transfer. This mutation placed a positively charged histidine at position 131, directly adjacent to positively charged lysines at positions 132 and 133, and very near lysine 91. The maximal values of $R_{\text{H}_2\text{O}}$ and k_{cat} for Y131H CA Vc were substantially reduced from wild type and Y131A CA Vc enzymes. The values for k_{cat}/K_M from stopped flow were also much lower than observed for all of the enzymes tested in this study.

This may be an indication that the placement of a histidine at position 131 in CA V caused a significant change in the environment of the active site cavity which affected the interconversion of CO_2 and HCO_3^- .

When examined by ^{18}O exchange at equilibrium, the k_{cat}/K_M pH profiles for all of the mutants were similar to wild type CA Vc. This is an indication that the rate of interconversion of CO_2 and HCO_3^- was not significantly altered, supporting the assumption that none of the mutations disrupted the overall structure and function of carbonic anhydrase. However, the pH profiles of k_{cat}/K_M determined at steady state by stopped flow were not unchanged. Y64A CA Vc and Y131H CA Vc had lower k_{cat}/K_M values than seen for the other enzymes. It appears that the interconversion of CO_2 and HCO_3^- at steady state in CA V is affected by these mutations. This is not unprecedented, pH profiles of k_{cat}/K_M for R67H HCA III are 10-fold greater than those of wild type HCA III (Ren *et al.*, 1995).

The inconsistency of k_{cat}/K_M from stopped flow compared to ^{18}O exchange is unusual. The value of R_1 , the rate of exchange of HCO_3^- and CO_2 obtained by ^{18}O exchange, has a substrate dependence described by equation

$$R_1/[E] = k_{\text{cat}}^{\text{ex}}[S]/(K_{\text{eff}}^S + [S]) \quad (9)$$

nine, in which $k_{\text{cat}}^{\text{ex}}$ is a rate constant for maximal interconversion of CO_2 and HCO_3^- ; K_{eff}^S is an apparent substrate binding constant, and $[S]$ is the

concentration of substrate, CO_2 or HCO_3^- (Simonsson *et al.*, 1979). In theory and in practice, $k_{\text{cat}}^{\text{ex}}/K_{\text{eff}}^{\text{CO}_2}$ is equal to $k_{\text{cat}}/K_{\text{M}}$ for hydration obtained by steady-state methods when $[\text{CO}_2]$ is much less than $K_{\text{eff}}^{\text{CO}_2}$ (Simonsson *et al.*, 1979). The lack of equivalence seen in CA V may be due to experimental biases that were not noticeable in previous studies. Unlike isozymes I and II of carbonic anhydrase, there is a large difference in the shape of the pH profiles for k_{cat} and $k_{\text{cat}}/K_{\text{M}}$ for the hydration of CO_2 by CA V. The effects of a histidine with a pK_a very near the pK_a of the zinc bound water in CA II may be much less noticeable upon the pH profile of $k_{\text{cat}}/K_{\text{M}}$ than the effects of a residue with a pK_a 2 pH units higher than the zinc bound water as found in CA V.

CHAPTER 6 DISCUSSION AND CONCLUSIONS

The purification and amino terminal analysis of carbonic anhydrase from mouse liver mitochondria was used to show that the cDNA sequence of Amor-Gueret and Levi-Strauss (1990) encoded the mouse homolog of carbonic anhydrase V. Two possible mature forms of CA V were purified from mitochondrial lysates; a truncated form that lacks a putative mitochondrial leader sequence, and an even shorter form that also lacks a region homologous to the initial 22 or 23 residues found in CA I, II, and III. The shorter form was within one residue of the amino termini determined for rat and guinea pig mitochondrial CA isolates (Hewett-Emmett *et al.*, 1986; Ohlinger *et al.*, 1993). Ngao *et al.* (1993; 1994) have demonstrated that human, rat, and mouse CA V are imported into the mitochondria of COS cells, and proteolytically truncated resulting in amino termini that correspond to positions -1 to 3 relative to the position numbering of CA I. Due to the susceptibility of the amino terminus of CA V to proteolysis, conclusive evidence for the actual amino terminus of the mature form of this protein as it exists in the mitochondria remains to be found. An *in vitro* mitochondrial

import assay coupled with an *in vitro* translation system might be a useful technique for determining how CA V is imported and processed in the mitochondrion. The *in vitro* translation system would have to be used to circumvent the proteolytic degradation of the amino terminus seen for full length forms of CA V expressed in bacterial cells.

Recombinant forms of mouse CA V corresponding to both possible mature forms were expressed in a bacterial system and shown to be active carbonic anhydrase enzymes with nearly identical catalytic properties. The expression of recombinant CA V also provided the first source of appreciable quantities of CA V for kinetic analysis and X-ray crystallography. This work describes the first kinetic characterization of CA V. The properties of the recombinant CA V enzymes were shown to be similar with the crude measurements of CA V activity and inhibition previously determined (reviewed by Dodgson, 1991a). The catalytic properties of CA V were investigated in detail, indicating that CA V is a high activity isozyme of carbonic anhydrase with maximal rates of catalysis similar to those for CA I and about 20% of CA II, one of the fastest enzymes known. CA V has an unusual sensitivity to pH and doesn't reach a maximal rate of hydration of CO_2 until the pH is well above 9. The pH profiles of the catalytic rate constants k_{cat} and $k_{\text{cat}}/K_{\text{M}}$ suggested that

the unusual pH sensitivity of CA V was due to a proton transfer pathway different from that of CA I or II.

Primary sequence comparisons as well as the crystal structure of mouse CA V suggested that proton transfer might require the tyrosine residues at positions 64 or 131 to act as proton acceptors. Site specific mutagenesis of both of these positions to alanines failed to significantly alter the catalytic properties of CA V, indicating that the tyrosines at position 64 and 131 were not necessary components of a possible proton transfer pathway. This was surprising since position 64 has been shown to play a significant role in proton transfer in CA II and III (Jewel *et al.*, 1991).

However, position 64 can be made into a useful proton transfer site by introducing a histidine at position 64 and substituting an alanine for the phenylalanine at position 65. The Y64H/F65A CA Vc double mutant had a maximal turnover number that was twice the value obtained for the wild type enzyme at high pH and 80-fold enhanced at pH 7. The enhanced catalytic rates of the Y64H/F65A CA V mutant support the hypothesis of Boriack *et al.* (1995) that phenylalanine 65 sterically hinders the access of a proton transfer residue at position 64 to the zinc-bound water in the active site. This is the first example of steric interference of proton transfer in carbonic anhydrases.

The purified CA V, in a collaboration with Dave Christianson's laboratory at the University of Pennsylvania, was used to determine the X-ray crystal structure of CA V. The crystal structure of mouse CA V is very similar to the structure determined for human CA II (Boriack *et al.*, 1995; Hakansson *et al.*, 1992). CA II and CA V have similar inhibition properties (Table 4-2), providing further evidence that the structure of the active sites is similar. However, there is a striking difference in the characteristics of proton transfer and the residues which participate in proton transfer between CA V and CA II. Measurements of the rates of catalysis by CA V over a range of pHs showed that the turnover number, k_{cat} , was dependent upon the ionization of a residue with a pK_a greater than 9. A histidine at position 64 with a pK_a of 7 has been identified as the proton transfer site in CA II.

Mutagenesis was used to make CA V more like CA II in the Y64H/F65A CA Vc mutant in terms of sequence and overall activity. However, the pH profile of k_{cat} for Y64H/F65A CA Vc still exhibits an increase at high pH indicative of a group involved in proton transfer with a $\text{pK}_a = 9$. This residue has not been identified, although several candidates have been ruled out (histidine 7, tyrosine 64, and tyrosine 131). The decrease in k_{cat} and $R_{\text{H}_2\text{O}}$ values determined for Y131H CA Vc may be an indication that this mutation disturbed other residues necessary for proton transfer. Three of the remaining

proton shuttle candidates, lysine 91, lysine 132, and lysine 133, are all located in close proximity to tyrosine 131. Of these three candidates, lysine 132 is most likely to be able to participate in proton transfer due to its high solvent accessibility. Several additional mutants of CA V will need to be studied in order to positively identify the residue(s) involved in proton transfer from the active site. Single alanine substitutions for the lysines at positions 132, 133, and 91 should be studied for their effects upon pH profiles of k_{cat} and $P_{\text{H}_2\text{O}}$. The possibility of multiple proton transfer pathways in CA V must also be considered. The Y64H/F65A CA V mutant demonstrated that more than one residue can function as a proton transfer site. To demonstrate the elimination of all proton transfer sites may require multiple mutations at positions 64, 131, 132, 133, and 91. The substitution of ionizable residues such as histidine or cysteine at these positions may also provide some insight about proton transfer.

The unique properties of carbonic anhydrase V found in this study lead to some speculations about why a specific carbonic anhydrase with these properties is needed in the mitochondria of the liver. Based upon their studies of a carbonic anhydrase activity in mitochondria, Dodgson *et al.* (1980) proposed that CA V may be necessary to provide HCO_3^- for the synthesis of carbamoyl phosphate in the mitochondrion. However, the rate of consumption

of HCO_3^- needed in the synthesis of urea, which requires carbamoyl phosphate for the formation of citrulline from ornithine en route to urea, was thought to be less than the uncatalyzed hydration of CO_2 (Dodgson and Forster, 1986). Inhibition of CA activity with ethoxzolamide did decrease urea synthesis in mitochondria (Dodgson and Forster, 1986). CA V is also thought to provide HCO_3^- for pyruvate carboxylase, a gluconeogenic enzyme (Dodgson, 1991a).

If the role of CA V in the mitochondria of the liver and kidney is to provide HCO_3^- for the synthesis of components of ureagenesis and gluconeogenesis, why is its maximal rate not achieved until well past a pH of 9? While the pH of the matrix of an actively respiring mitochondrion is expected to be alkaline, most estimates place this internal pH between 8 and 9 (Mitchell, 1979). The large change in activity of CA V over this pH range suggests that CA V may play a role in processes that do not require substantial CA activity at pH values less than 8. Since the rate of uncatalyzed hydration of CO_2 increases at higher pH, supplying HCO_3^- does not fit this line of reasoning. Storey and Forster (1990) have suggested that CA V may be important in the mediation of mitochondrial volume changes by providing matrix HCO_3^- as counterion for K^+ influx to increase matrix volume. Swelling of the liver mitochondrial matrix increases pyruvate carboxylation and citrulline

synthesis (reviewed by Halestrap, 1989). Further evidence for this hypothesis might be found by examining the effect of sulfonamide inhibitors upon other processes which are dependent upon volume changes in mitochondria.

REFERENCES

- Amor-Gueret, M., and Levi-Strauss, M. (1990) *Nucleic Acids Res.* **18**, 1646.
- Aronsson, G., Mårtensson, L., Carlsson, U., and Jonsson, B.-H. (1995) *Biochemistry* **34**, 2153-2162.
- Beaufay, H., Bendall, D.S., Baudhuin, P., and De Duve, C. (1959) *Biochem J.* **17**, 623-628.
- Beavis, A.D., Garlid, K.D. (1987) *J. Biol. Chem.* **262**, 15085-15093.
- Bell, R.P. (1973) The Proton in Chemistry (Cornell University Press, Ithaca, NY) Chapter XI, 191-225.
- Boriack-Sjodin, P.A., Heck, R.W., Laipis, P.J., Silverman, D.N., and Christianson, D.W. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 10949-10953.
- Coulson, R.A., and Hebert, J.D. (1984) *Ann. N.Y. Acad. Sci.* **429**, 505-515.
- Datta, P.K., and Shepard, T.H. (1959) *Arch. Biochem. Biophys.* **81**, 124-129.
- Dixon, T.F., and Purdom, M. (1954) *J. Clin. Pathol.* **7**, 341-343.
- Dodgson, S.J. (1991a) The Carbonic Anhydrases Ed. Dodgson, S.J., Tashian, R.E., Gros, G., and Carter, N.D. (Plenum Press, New York) 297-306.
- Dodgson, S.J. (1991b) *Biochem. Cell. Biol.* **69**, 761-763.
- Dodgson, S.J., and Cherian, K. (1989) *Amer. J. Physiol.* **257**, E791-E796.
- Dodgson, S.J., and Forster, R.E. (1986) *J. Appl. Physiol.* **60**, 646-652.
- Dodgson, S.J., Forster, R.E., Schwed, D.A., and Storey, B.T. (1983) *J. Biol. Chem* **258**, 7696-7701.
- Dodgson, S.J., Forster, R.E., Storey, B.T. (1982) *J. Biol. Chem.* **257**, 1705-1711.

- Dodgson, S.J., Forster, R.E., Storey, B.T., and Mela, L. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5562-5566.
- Dodgson, S.J., and Watford, M. (1990) *Arch. Bioch. Biophys.* **277**, 410-414.
- Eriksson, E.A. (1988) Structural differences between high and low forms of carbonic anhydrases, Doctoral dissertation, Uppsala University.
- Eriksson, E.A., Jones, T.A., and Liljas, A. (1986) *Zinc Enzymes* Ed. Bertini, I., Luchinat, C., Maret, W., and Zeppezaur, M. (Birkhäuser, Boston) 317-327.
- Fiske, C.H., SubbaRow, Y. (1925) *J. Biol. Chem.* **66**, 375-381.
- Glasoe, P.K., and Long, F.A. (1960) *J. Phys. Chem* **64**, 188-191.
- Gros, G., and Dodgson, S.J. (1988) *Annu. Rev. Physiol.* **50**, 669-694.
- Hakansson, K., Carlsson, M., Svensson, L.A., and Liljas, A. (1992) *J. Mol. Biol.* **227**, 186-190.
- Halestrap, A.P. (1989) *Biochim. Biophys. Acta* **973**, 355-382.
- Hartl, F.U., Pfanner, N., Nicholson, D.W., and Neupert, W. (1989) *Biochim. Biophys. Acta.* **988**, 1-45.
- Häussinger, D., Kaiser, S., Stehle, T., and Gerok, W. (1986) *Biochem. Pharmacol.* **35**, 3317-3322.
- Heck, R.W., Tanhauser, S.M., Manda, R., Tu, C., Laipis, P.J., Silverman, D.N. (1994) *J. Biol. Chem.* **269**, 24742-24746.
- Hendrick, J.P., Hodges, P.E., and Rosenberg, L.E. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 4056-4060.
- Hewett-Emmett, D., Cook, R.G., and Dodgson, S.J. (1986) *Isozyme Bulletin*, **19**, 13.
- Hölmes, R.S. (1977) *Eur. J. Biochem.* **78**, 511-520.
- Jewel, D.A., Tu, C.K., Paranaewithana, S.R., Tanhauser, S.M., LoGrasso, P.V., Laipis, P.J., and Silverman, D.N. (1991) *Biochemistry* **30**, 1484-1490.
- Karhukorpi, E.-K., Lakkakorpi, P., Carter, N., Dodgson, S., and Väänänen, K. (1992) *Histochem. J.* **24**, 9-14.

- Khalifah, R.G. (1971) *J. Biol. Chem.* **246**, 2561-2573.
- Khalifah, R.G. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 2986-2991.
- Khalifah, R.G., Strader, D.J., Bryant, S.H., and Gibson, S.M. (1977) *Biochemistry* **16**, 2241-2247.
- Kunkel, T.A., Roberts, J.D., and Zakour, R.A. (1987) *Methods in Enz.* Ed. Wu, R., and Grossman, L. **154**, 367-382.
- Lansman, R.A., Shade, R.O., Shapira, J.F., and Avise, J.C. (1981) *J. Mol. Evol.* **17**, 214-226.
- Lindskog, S., and Coleman, J.E. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 2505-2510.
- Lindskog, S., Engberg, P., Forsman, C., Ibrahim, S.A., Jonsson, B.H., Simonsson, I., and Tibell, L. (1984) *Ann. N.Y. Acad. Sci.* **429**, 62-75.
- Marchuk, D., Drumm, M., Sauolino, A., and Collin, F.S. (1991) *Nucleic Acids Res.* **19**, 1154.
- Maren, T.H., and Ellison, A.C. (1967) *Molec. Pharmacol.* **3**, 503-508.
- Meldrum, N.U., and Roughton, F.J.W. (1933) *J. Physiol.* **80**, 113-142.
- Mitchell, P. (1979) *Science* **206**, 1148-1159.
- Ngao, Y., Platero, J.S., Waheed, A., and Sly, W.S. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 7623-7627.
- Ngao, Y., Srinivasan, M., Platero, J.S., Svendrowski, M., Waheed, A., and Sly, W.S. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 10330-10334.
- Nyman, P.O. (1961) *Biophys. Acta.* **53**, 1-12.
- Ohlinger, D.E., Lynch, C.J., Forster, R.E., and Dodgson, S.J. (1993) *FASEB Journal* **7**, A676.
- Ohnishi, S.T., and Barr, J.K. (1978) *Anal. Biochem.* **86**, 193-197.
- Pocker, Y., and Bjorkquist, D.W. (1977) *Biochemistry* **16**, 5698.
- Ren, X., Tu, C.K., Laipis, P.J., and Silverman, D.N. (1995) *Biochemistry* **34**, 8492-8498.

- Sanyal, G., Swenson, E.R., Pessah, N.I., and Maren, T.H. (1982) *Mol. Pharmacol.* **22**, 211-220.
- Segel, I.H. (1975) *Enzyme Kinetics*, pp. 150-159, John Wiley & Sons, Inc., New York.
- Silverman, D.N. (1982) *Methods Enzymol.* **87**, 732-752.
- Simonsson, I., Jonsson, B.-H. and Lindskog, S. (1979) *Eur. J. Biochem.* **93**, 409-417.
- Simonsson, I., and Lindskog, S. (1982) *Eur. J. Biochem.* **123**, 29-36.
- Stadie, W.C., and O'Brien, H. (1933) *J. Biol. Chem.* **103**, 521-529.
- Steiner, H., Jonsson, B.H., and Lindskog, S. (1975) *Eur. J. Biochem.* **59**, 253-257.
- Storey, B.T., and Forster, R.E. (1991) Carbonic Anhydrase Ed. Botré, F., Gros, G., and Storey, B.T. (VCH, Publishers, New York) 299-310.
- Swanson, M.A. (1955) *Methods in Enzymol.* **2**, 541-543.
- Tanhauser, S.M., Jewell, D.A., Tu, C.K., Silverman, D.N., and Laipis, P.J. (1992) *Gene (Amst.)* **117**, 113-117.
- Tashian, R.E. (1989) *Bioessays* **10**, 186-192.
- Tashian, R.E., Hewett-Emmett, D., and Venta, P.J. (1991) Carbonic Anhydrase Ed. Botré, F., Gros, G., and Storey, B.T. (VCH, Publishers, New York) 151-161.
- Tu, C.K., and Silverman, D.N. (1982) *Biochemistry* **21**, 6353-6360.
- Tu, C.K., and Silverman, D.N. (1989) *Biochemistry* **28**, 7913-7918.
- Tu, C.K., Wynns, G.C., and Silverman, D.N. (1981) *J. Biol. Chem.* **256**, 9466-9470.
- Väänänen, K., Carter, N.D., and Dodgson, S.J. (1991) *J. Histochem. Cytochem.* **39**, 451-459.

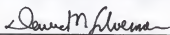
Wroblewski, F., and LaDue, J.S. (1955) *Proc. Soc. Exp. Biol. Med.* **90**, 210-212.

BIOGRAPHICAL SKETCH

Robert (Bob) Wilson Heck was born on April 8, 1966, in Cincinnati, Ohio. During his teenage years, he relocated to Palm Beach Gardens, Florida, and graduated from Palm Beach Gardens High School in 1984. He went to Gainesville to attend the University of Florida the same year.

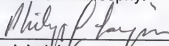
During his undergraduate years, Bob developed an interest in science and research. Ultimately, he chose biochemistry as his major area of study. In 1987, he began his involvement in biochemistry by undertaking an undergraduate research project. In the spring of 1988, he graduated with high honors and obtained a Bachelor of Science degree in biochemistry. That same year he entered graduate school at the University of Florida in the Department of Biochemistry and Molecular Biology.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



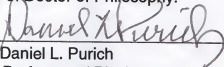
David N. Silverman, Chairman
Professor of Pharmacology
and Therapeutics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



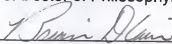
Philip J. Laipis
Professor of Biochemistry
and Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



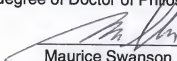
Daniel L. Purich
Professor of Biochemistry
and Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Brian Cain
Associate Professor of Biochemistry
and Molecular Biology

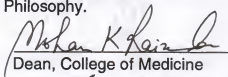
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

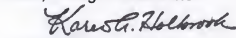


Maurice Swanson
Associate Professor of Molecular
Genetics and Microbiology

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 1996


Dean, College of Medicine


Dean, Graduate School

UNIVERSITY OF FLORIDA



3 1262 08554 7361